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THE COMPARISON OF THE EFFICIENCY OF ANTHELMINTICS

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ANY comparison of the efficiency of drugs as anthelmintics is the relationship between an *a priori* finding of a certain degree of infestation in a sample of individuals and the *a posteriori* finding of another degree of infestation in either the same individuals or a sample of similarly infested individuals to which the drug has been administered. That both the *a priori* and the *a posteriori* degree of infestation can be determined is a *sine qua non* of such a method. In such an investigation the following assumptions pertain:

(1) All members of the species of host of similar age and exposed to a similar risk of infestation are equally susceptible to infestation by the helminth.

(2) All the helminth parasites harboured by an individual host are equally exposed to, and susceptible to, the action of the drug or mixture of drugs which is being tested.

(3) Reliable laboratory methods are at hand for the determination of the initial infestation of the untreated animals and the residual infestation of the treated animals.

It is not within the province of this paper to discuss or consider any practical difficulties which may be encountered by workers desirous of investigating the efficiency of drugs or mixtures of drugs as specifics against a given species of helminth in a given host. The onus is upon them to adopt the best technique.

Seddon and Ross (1928) determined the mean number of the specific helminth, $\bar{X}$, which was found to be harboured, post-mortem, by a random sample of hosts of similar age, maintained reputedly in an environment in which all individuals were exposed to a similar risk of infestation. From the same population of hosts another sample was selected and treated with a drug or mixture of drugs. The mean number of the specific helminth, $\bar{Y}$, remaining in the treated animals was now determined. Their method of expression of the efficiency of the treatment, E, was:

$$E = \frac{100\,(\bar{X} - \bar{Y})}{\bar{X}} \qquad \text{.....(1).}$$

Roberts (1932) has adopted precisely similar methods in his investigations.

These workers obtained, as the expression of the efficiency of the several drugs or mixtures of drugs tested, a bald figure in which no consideration is given to the variation in the degree of infestation which was found to exist in their untreated animals. They are, therefore, unable to interpret the observed differences in the relative efficiency between the drugs or mixtures of drugs.

THEORETICAL CONSIDERATIONS

Let $X_1, X_2, X_3, \ldots, X_s$ be the numbers of the specific helminth, found to be harboured by s individual hosts, from a population of equally susceptible hosts of similar age, and exposed to a similar risk of infestation. Let $\overline{X}$ be the mean of this series of observations, and let σ_X be the standard deviation of $\overline{X}$. Also let $v_X = \frac{\sigma_X}{\overline{X}}$. Further let $Y_1, Y_2, Y_3, \ldots, Y_p$ be the numbers of the specific helminth, found to be harboured by p individual hosts from the same population which have received some form of treatment. Let $\overline{Y}$ be the mean of this series of observations and let σ_Y be the standard deviation of Y. Further, let $v_Y = \frac{\sigma_Y}{\overline{Y}}$.

It is suggested that the best estimate of the relationship which exists between these two infestations is to be obtained by comparing the residual infestation of each of the treated hosts with the initial infestation of each of the untreated hosts. Thus the following series of ratios or indices is formed:

$$\begin{array}{cccccc}
\frac{Y_1}{X_1} & \frac{Y_2}{X_1} & \frac{Y_3}{X_1} & \cdots & \frac{Y_p}{X_1} \\
\frac{Y_1}{X_2} & \frac{Y_2}{X_2} & \frac{Y_3}{X_2} & \cdots & \frac{Y_p}{X_2} \\
\cdots\cdots & \cdots\cdots & \cdots\cdots & \cdots & \cdots\cdots \\
\frac{Y_1}{X_{s-1}} & \frac{Y_2}{X_{s-1}} & \frac{Y_3}{X_{s-1}} & \cdots & \frac{Y_p}{X_{s-1}} \\
\frac{Y_1}{X_s} & \frac{Y_2}{X_s} & \frac{Y_3}{X_s} & \cdots & \frac{Y_p}{X_s}
\end{array}$$

Let E be the mean of this series of ps ratios and σ_E be the estimate of the standard deviation of E. Then it can be readily shown that:

$$E = \frac{1}{ps} \sum_1^p (Y_1) . \sum_1^s \left(\frac{1}{X_1}\right) \qquad \ldots\ldots(2),$$

and

$$\sigma_E^2 = \frac{1}{(ps-1)} \left\{ \sum_1^p (Y_1)^2 \sum_1^s \left(\frac{1}{X_1}\right)^2 - ps . E^2 \right\} \qquad \ldots\ldots(3).$$

Those to whom the symbolic notation may be confusing are referred to the practical applications of these formulae (see pp. 469 and 470).

There exists an alternative form of expression of both E and σ_E in terms of the constants of the parent distributions, and each of these is of some theoretical importance. We shall give these expressions in terms of the first six moments of the frequency distribution of the series $X_1, X_2, \ldots, X_s$, and of those moments of the frequency distribution of the series $Y_1, Y_2, \ldots, Y_p$ which may enter.

By simple algebraic expansion it can be shown that:

$$E=\frac{\overline{Y}}{\overline{X}}\left\{1+\frac{1}{\overline{X}p^2}\Sigma(X_1)^2-\frac{1}{\overline{X}p^3}\Sigma(X_1)^3+\frac{1}{\overline{X}p^4}\Sigma(X_1)^4-\frac{1}{\overline{X}p^5}\Sigma(X_1)^5+\frac{1}{p\overline{X}^6}\Sigma(X_1)^6\right\} \quad \text{.....(4),}$$

and $$\sigma_E^2=\frac{\overline{Y}^2}{\overline{X}^2}\left\{1+\frac{1}{s\overline{Y}^2}\Sigma(Y_1)^2\right\}\left\{1+\frac{3}{p\overline{X}^2}\Sigma(X_1)^2-\frac{4}{p\overline{X}^3}\Sigma(X_1)^3+\frac{s}{p\overline{X}^4}\Sigma(X_1)^4 -\frac{6}{p\overline{X}^5}\Sigma(X_1)^5+\frac{7}{p\overline{X}^6}\Sigma(X_1)^6\right\}-E^2 \quad \text{.....(5).}$$

It should be noted, however, that whereas the value of σ_E defined by equation (3) is based only on $(ps-1)$ degrees of freedom, that defined by equation (5) is based on ps degrees of freedom.

The first thing to note in equation (4) is that E is entirely independent of all constants of the series $Y_1, Y_2, Y_3, \ldots, Y_p$, except the mean or first moment. Thus the mean value of the efficiency of the treatment is entirely independent of the variation found in the residual infestation of the selected and treated hosts. Also E will approximate to $\overline{Y}/\overline{X}$ as the expression within the brackets tends to unity.

σ_E is dependent upon the magnitude of the first two moments of the distribution of $Y_1, Y_2, Y_3, \ldots, Y_p$ and all the moments of the distribution of $X_1, X_2, X_3, \ldots, X_s$.

Should, however, the initial infestation of the hosts be distributed according to the normal law of error, the approximations given by equations (4) and (5) become very much less imposing, when it is remembered that in any series obeying the normal law, all odd moments do not differ significantly from zero, and that $\beta_2 \to 3$ and $\beta_4 \to 15$, so that in such an instance

$$E=\frac{\overline{Y}}{\overline{X}}\{1+v_X^2+3v_X^4+15v_X^6\} \quad \text{.....(6)}$$

and $$\sigma_E^2=\frac{\overline{Y}^2}{\overline{X}^2}\{1+v_Y^2\}\{1+3v_X^2+15v_X^4+105v_X^6\}-E^2 \quad \text{.....(7).}$$

COMPARISON OF THE CRITERIA OF EFFICIENCY

We shall only consider the limits of the criteria which are likely to be encountered in efficiently conducted investigations. The criterion defined by Seddon and Ross will vary between zero and 100 for all efficiently conducted experiments; while the criterion suggested by this paper will vary from zero to unity for all efficiently conducted experiments. There is, however, a difference of interpretation. When E as defined by Seddon and Ross approaches 100, the efficiency of the anthelmintic approaches perfection, whereas such a state of efficiency is encountered when E (as defined in this paper) approaches zero. Similarly a totally inefficient anthelminthic, gives a value of E (defined by Seddon and Ross) of zero and a value of E (as defined in this paper) of unity.

Theoretically it is possible to obtain values of E (defined by Seddon and Ross) which are less than zero and values of E (as defined in this paper) which are greater than 1. In such cases $\overline{Y}$ is greater than $\overline{X}$; that is, the mean number of helminths infesting the treated animals is greater than the mean number of helminths infesting the untreated animals. Such a condition of affairs is impossible under the prescribed assumptions of the method of determining the efficiency of an anthelmintic.

Practical applications of the suggested criterion of the efficiency of an anthelmintic

We shall choose firstly the data submitted by Roberts (1932) and secondly portions of the data submitted by Seddon and Ross (1928) as material to which the suggested theoretical considerations may be applied.

Roberts dealt with two distinct populations of hosts for *Haemonchus contortus*, viz.:

(1) The first population was of lambs of "mixed sexes about five months old which had been together since birth". Thirty such lambs were selected and divided at random into seven groups:

(*a*) five groups, each contained five individuals,
(*b*) one group contained three animals,
(*c*) one group contained two animals.

(2) The second population was of "three to five year old wethers which had been running under the same conditions for the past three years". Forty such wethers were selected and divided at random into eight groups:

(*a*) one group contained eight individuals,
(*b*) five groups each contained five individuals,
(*c*) another group contained four individuals,
(*d*) another group contained three individuals.

The group containing four individuals is not considered in the following analysis.

The constants determined from the data are given in Tables I and II.

Table I. *The constants of the distribution of the number of* Haemonchus contortus *found in the groups selected as controls (Roberts' data)*

In Table I for each sample σ is estimated with $(n-1)$ degrees of freedom.

Population	No. of animals	Total No. of *H. contortus*	Mean No. of *H. contortus* per control $\overline{X}$	σ_X	v_X
Lambs	5	16,375	3275	650·02	0·20
Adults	8	6,425	803	506·23	0·63

The magnitude of v_X gives some indication of the variation of the individual degree of infestation; so that while it may be said that the average infestation of the lambs is fairly well determined, the same does not hold for the average infestation of the adults.

Table II. *The constants of the distribution of the residual number of* Haemonchus contortus *found in the treated groups of both populations (Roberts' data)*

In Table II for each sample σ is estimated with $(n-1)$ degrees of freedom.

Population	Treatment	No. of animals	Total No. of residual *H. contortus*	Mean No. of residual *H. contortus* $\bar{Y}$	σ_Y	v_Y
Lambs	Copper sulphate and mustard	5	2724	545	436·92	0·80
	1 c.c. carbon tetrachloride and 4 c.c. liquid paraffin	5	1468	294	323·84	1·10
	Tetrachlorethylene 2·5 c.c.	2	3799	1900	1082·58	0·57
	Tetrachlorethylene 5 c.c.	2	28	14	14·14	1·01
	Copper sulphate and sodium arsenite	5	5370	1074	1255·02	1·16
	Arsenic and magnesium sulphate	5	3013	603	120·21	0·20
Adults	Copper sulphate and mustard	5	850	170	184·53	1·09
	Carbon tetrachloride 2 c.c. and 3 c.c. liquid paraffin	5	640	128	80·23	0·63
	Carbon tetrachloride 5 c.c.	5	24	8	10·58	1·32
	Tetrachlorethylene 5 c.c.	5	1526	305	172·56	0·57
	Copper sulphate and sodium arsenite	5	529	106	112·06	1·06
	Arsenic and magnesium sulphate	5	740	148	167·43	1·13

It will be noted that with the exception of the group of lambs which received arsenic and magnesium sulphate, there is a very large variation in the residual number of *H. contortus* in the individuals of all treated groups.

THE CRITERIA OF EFFICIENCY OF THE ANTHELMINTICS

An example shall be given showing the manner in which the mean efficiency of the anthelmintic and its standard deviation can be determined.

Denote the mean efficiency by E, and its standard deviation by σ_E.

To calculate E from equation (2) *for lambs which received copper sulphate and mustard.*

There are five lambs in the untreated group and five lambs in the treated group; therefore $s=5$ and $p=5$, ps ratios are formed, i.e., 25 ratios are formed.

$$E=\frac{1}{ps}\left\{\sum_1^p (Y_1).\sum_1^s \left(\frac{1}{X_1}\right)\right\}$$

$$=\frac{1}{ps}\left\{\begin{matrix}\text{sum of the residual number}\\ \text{of } H.\ contortus \text{ in each of the}\\ \text{animals in the treated group}\end{matrix}\right\}\left\{\begin{matrix}\text{the sum of the reciprocals of the}\\ \text{number of } H.\ contortus \text{ in each of}\\ \text{the animals of the untreated group}\end{matrix}\right\} \quad \ldots\ldots(2),$$

$$\left\{\begin{matrix}\text{the sum of the residual number of}\\ H.\ contortus \text{ in each of the animals}\\ \text{of the treated group}\end{matrix}\right\}=\{214+315+1265+652+278\}=2724,$$

$$\left\{\begin{matrix}\text{the sum of the reciprocals of the}\\ \text{number of } H.\ contortus \text{ in each of}\\ \text{the animals of the untreated group}\end{matrix}\right\}=\left\{\frac{1}{3624}+\frac{1}{2646}+\frac{1}{2978}+\frac{1}{2886}+\frac{1}{4241}\right\}$$

$$=0{\cdot}0015720 \qquad \ldots\ldots(a).$$

$$\therefore\ E=\frac{1}{25}\times 2724\times 0{\cdot}0015720=0{\cdot}1713.$$

To calculate σ_E from equation (3) for lambs which received copper sulphate and mustard.

$$\sigma_E^2=\frac{1}{(ps-1)}\left\{\sum_1^p (Y_1)^2.\sum_1^s\left(\frac{1}{X_1}\right)^2-psE^2\right\} \qquad \text{.....(3),}$$

$$i.e.\ \sigma_E^2=\frac{1}{(ps-1)}\left\{\begin{bmatrix}\text{the sum of the}\\ \text{squares of the}\\ \text{residual number}\\ \text{of } H.\ contortus\\ \text{in each of the}\\ \text{animals of the}\\ \text{treated group}\end{bmatrix}\begin{bmatrix}\text{the sum of the}\\ \text{squares of the}\\ \text{reciprocals of } H.\\ contortus \text{ in each}\\ \text{of the animals}\\ \text{of the untreated}\\ \text{group}\end{bmatrix}-ps\begin{bmatrix}\text{the square of}\\ \text{the mean value}\\ \text{of the efficiency}\end{bmatrix}\right\}.$$

$$\begin{bmatrix}\text{The sum of the squares}\\ \text{of the residual number}\\ \text{of } H.\ contortus \text{ in each}\\ \text{of the animals of the}\\ \text{treated group}\end{bmatrix}=\{214^2+315^2+1265^2+652^2+278^2\}=2{,}247{,}634.$$

$$\begin{bmatrix}\text{The sum of the recipro-}\\ \text{cals of the squares of the}\\ \text{number of } H.\ contortus\\ \text{in each of the animals}\\ \text{of the untreated group}\end{bmatrix}=\left\{\frac{1}{3624^2}+\frac{1}{2646^2}+\frac{1}{2978^2}+\frac{1}{2886^2}+\frac{1}{4241^2}\right\}$$

$$=0{\cdot}000{,}000{,}5074 \qquad \text{.....(b),}$$

$$ps=25 \text{ and } (ps-1)=24, \text{ so that } ps\begin{Bmatrix}\text{the square of}\\ \text{the mean value}\\ \text{of the efficiency}\end{Bmatrix}=25\times 0{\cdot}1713^2=0{\cdot}7336.$$

$$\therefore\ \sigma_E^2=\frac{1}{24}\left\{2{,}247{,}634\times 0{\cdot}000{,}000{,}5074-0{\cdot}7336\right\}=\frac{0{\cdot}4068}{24}$$

$$=0{\cdot}0169.$$

$$\therefore\ \sigma_E=0{\cdot}1300.$$

It will be noted that for all anthelmintics tested upon samples of the same population, the factors denoted by (*a*) and (*b*) will be used as multipliers in the determination of E and σ_E. Therefore once (*a*) and (*b*) are determined, it is a matter only of simple arithmetic to determine all the required values of E and σ_E. No tedious computations are required and a valuable aid would be Barlow's *Tables of Squares, Cubes and Reciprocals.*

Table III. *The estimates of the mean efficiency of the drugs and mixtures of drugs, as specifics against* Haemonchus contortus *in sheep (Robert's data)*

Treatment	Population: Lambs ps	Lambs E_L	Lambs σ_{E_L}	Adults ps	Adults E_A	Adults σ_{E_A}
(1) Copper sulphate and mustard	25	0·1713	0·1300	40	0·3206	0·4637
(2) Carbon tetrachloride 1 c.c.	25	0·0923	0·1338	—	—	—
(3) ,, 2 c.c.	—	—	—	40	0·2414	0·2526
(4) ,, 5 c.c.	—	—	—	24	0·0151	0·0202
(5) Tetrachlorethylene 2·5 c.c.	10	0·5972	0·2768	—	—	—
(6) ,, 5·0 c.c.	10	0·0044	0·0036	40	0·5756	0·5711
(7) Copper sulphate and sodium arsenite	25	0·3377	0·3692	40	0·1995	0·2835
(8) Arsenic and magnesium sulphate	25	0·1895	0·0469	40	0·2791	0·4261

σ_{E_L} and σ_{E_A} are calculated for $(ps - 1)$ degrees of freedom.

If one now wishes to compare cursorily the estimate of the mean efficiency of any drug or mixture of drugs, as specific against *H. contortus*, as given in Table III, with that stated by Roberts, it is necessary to subtract the tabulated value of E from unity and multiply the result by 100. It will be noticed that for the lambs the estimates of the efficiency, as defined either by myself or Seddon and Ross, are sensibly identical except in the case of arsenic and magnesium sulphate (see Roberts (1932), Table 8, p. 14). Here the mean efficiency may be expressed as 80·05 per cent., as against 87 per cent. given by Roberts. Should, however, this method of comparison be employed for adults, it will be found that with the exception of carbon tetrachloride at a dose rate of 5 c.c. the mean value of the efficiency of any drug or mixture of drugs as given by the method herein suggested, is appreciably lower than the value of the efficiency stated by Roberts. The difference becomes very marked in the case of tetrachlorethylene at a dose rate of 5 c.c. to adult sheep, where we find 42·44 per cent. mean efficiency as compared with 62 per cent. stated by Roberts. It is quite obvious by reference to equation (4) that when the effect of variation of the individual infestation of the control animals is taken into account, the mean efficiency will be lower than an estimate of the efficiency in which no heed is paid to such variation.

THE COMPARISON OF THE EFFICIENCY OF THE DRUGS OR MIXTURE OF DRUGS AS SPECIFICS AGAINST *HAEMONCHUS CONTORTUS* IN SHEEP

It has been previously pointed out that it was impossible to compare logically the various estimates of the efficiency of any drug when the efficiency was defined by Seddon and Ross (1928). From the data of Table III a logical comparison of each drug or mixture of drugs for a given population can be made.

It is not sufficient, however, to say, for instance, that arsenic and magnesium sulphate, when administered to lambs, was more efficient as a specific

against *H. contortus*, than copper sulphate and sodium arsenite by 0·1482. It is necessary to determine how often could such a difference be obtained by the operation of chance alone, were similar experimental conditions to pertain during many repetitions of the experiment[1].

The essential constants for the judgment of the significance of the difference between the mean efficiency of the several drugs are set out in Table IV.

The numerals in brackets in Table III, are used as prefixes to denote the values of E which are being compared.

Table IV. *The significance of the difference between the mean efficiency of the drugs and mixtures of drugs, tested by Roberts, as specifics against* Haemonchus contortus *in sheep*

Nomenclature of difference	Magnitude of difference	t		P	Remarks
Lambs:					
${}_1E_L - {}_6E_L$	0·1669	4·0041		$1·0 \times 10^{-4}$	Difference significant
${}_2E_L - {}_6E_L$	0·0879	2·0517		0·044	,,
${}_5E_L - {}_6E_L$	0·5928	9·5545	Less than	$1·0 \times 10^{-9}$	,,
${}_7E_L - {}_6E_L$	0·3333	2·8270	Between	$1·0 \times 10^{-2}$ and $1·0 \times 10^{-3}$	,,
${}_8E_L - {}_6E_L$	0·1851	12·0072	Less than	$1·0 \times 10^{-9}$	,,
${}_1E_L - {}_2E_L$	0·0790	2·9947	Between	$1·0 \times 10^{-2}$ and $1·0 \times 10^{-3}$	,,
${}_5E_L - {}_2E_L$	0·4949	7·1845	Less than	$1·0 \times 10^{-9}$	,,
${}_7E_L - {}_2E_L$	0·2454	4·4184		$1·0 \times 10^{-5}$	,,
${}_8E_L - {}_2E_L$	0·0972	4·8600		$1·0 \times 10^{-6}$	,,
${}_5E_L - {}_1E_L$	0·4159	6·0906		$1·0 \times 10^{-9}$	,,
${}_7E_L - {}_1E_L$	0·1664	3·0057	Between	$1·0 \times 10^{-2}$ and $1·0 \times 10^{-3}$	,,
${}_8E_L - {}_1E_L$	0·0182	0·9286		0·35	Difference not significant
${}_5E_L - {}_8E_L$	0·3977	7·0860		$1·0 \times 10^{-9}$	Difference significant
${}_7E_L - {}_8E_L$	0·1482	2·8153	Between	$1·0 \times 10^{-2}$ and $1·0 \times 10^{-3}$	,,
${}_7E_L - {}_5E_L$	0·2495	1·9250		0·051	Difference almost significant
Adults:					
${}_1E_A - {}_4E_A$	0·3055	3·2178	Nearly	$1·0 \times 10^{-3}$	Difference significant
${}_3E_A - {}_4E_A$	0·2263	4·3163		$1·0 \times 10^{-5}$	,,
${}_6E_A - {}_4E_A$	0·5605	4·7921		$1·0 \times 10^{-5}$	,,
${}_7E_A - {}_4E_A$	0·1844	3·1756	Nearly	$1·0 \times 10^{-3}$	,,
${}_8E_A - {}_4E_A$	0·2640	3·0984	Between	$1·0 \times 10^{-2}$ and $1·0 \times 10^{-3}$	,,
${}_1E_A - {}_7E_A$	0·1211	1·4092		0·16	Difference not significant
${}_3E_A - {}_7E_A$	0·0419	0·6945		0·48	,,
${}_6E_A - {}_7E_A$	0·3761	3·7063		$1·0 \times 10^{-3}$	Difference significant
${}_8E_A - {}_7E_A$	0·0796	0·9935		0·32	Difference not significant
${}_1E_A - {}_3E_A$	0·0792	0·9485		0·34	,,
${}_6E_A - {}_3E_A$	0·3342	3·3844		$1·0 \times 10^{-3}$	Difference significant
${}_8E_A - {}_3E_A$	0·0377	0·5045		0·61	Difference not significant
${}_1E_A - {}_8E_A$	0·0415	0·4213		0·67	,,
${}_6E_A - {}_8E_A$	0·2965	2·6367	Between	$1·0 \times 10^{-2}$ and $1·0 \times 10^{-3}$	Difference significant
${}_6E_A - {}_1E_A$	0·2550	2·1921		0·029	,,

A careful survey of Table IV may impress upon those who are often given to such a procedure, the danger of asserting significance between the differences

[1] The analytical methods used in such comparisons are those evolved by "Student" and brought into common usage by Dr R. A. Fisher (1930) and known as the "t" test. The maximum value of P for which a difference between any two means shall be considered significant will be 0·05: thus when the odds against the chance occurrence of such a difference are equal to or greater than 20 to 1, significance will be asserted.

of two means without the application of efficient methods. For instance, such persons may state that for lambs this data demonstrates that a mixture of copper sulphate and mustard is a significantly more efficient specific against *H. contortus* than is tetrachlorethylene at a dose rate of 2·5 c.c. (*vide* ${}_7E_L - {}_5E_L$). Such a statement is scarcely supported by the available evidence. They also may not consider that carbon tetrachloride at a dose rate of 1 c.c. in 4 c.c. liquid paraffin is a more efficient specific against *H. contortus* in lambs than is a mixture of arsenic and magnesium sulphate (*vide* ${}_8E_L - {}_2E_L$), but such an inference would be erroneous.

CONCLUSIONS FROM ROBERTS' DATA

In so far as one may consider Roberts' data to have fulfilled the requirements essential to the efficient determination of the efficacy of various drugs or mixtures of drugs as specifics against *H. contortus* in sheep, the following are the permissible conclusions:

A. *For lambs*

(1) Tetrachlorethylene at a dose rate of 5 c.c. is a significantly more efficient specific against *H. contortus* than is any other drug or mixture of drugs tested. Tetrachlorethylene at this dose rate is just significantly more efficient than carbon tetrachloride at a dose rate of 1 c.c. in 4 c.c. liquid paraffin.

(2) Carbon tetrachloride at a dose rate of 1 c.c. in 4 c.c. liquid paraffin is a significantly more efficient specific against *H. contortus* than any other drug or mixture of drugs tested (except tetrachlorethylene at a dose rate of 5 c.c.).

(3) A mixture of copper sulphate and mustard is a more efficient specific against *H. contortus* than is either tetrachlorethylene at a dose rate of 2·5 c.c. or a mixture of copper sulphate and sodium arsenite.

(4) There is no difference between arsenic and magnesium sulphate and copper sulphate and mustard as specifics against *H. contortus*.

(5) While it would appear that both tetrachlorethylene at a dose rate of 2·5 c.c. and copper sulphate and sodium arsenite do exert some specific effect in the reduction of the infestation by *H. contortus*, such a specific action at the dose rate used is significantly less than that observed for other drugs or mixtures of drugs.

B. *For adults*

(1) Carbon tetrachloride at a dose rate of 5 c.c. is significantly more efficient as a specific against *H. contortus* than is any other drug or mixture of drugs tested.

(2) Tetrachlorethylene at a dose rate of 5 c.c. is significantly *less* efficient as a specific against *H. contortus* than is any other drug or mixture of drugs which was tested.

(3) There is no significant difference between the other drugs or mixtures of drugs which were used as specifics against *H. contortus*.

Seddon and Ross' data

Certain portions of Exp. No. 6 of these workers will be considered, namely the investigation of the efficiency of tetrachlorethylene at a dose rate of 2·5 c.c. for lambs and 1 c.c. carbon tetrachloride and 4 c.c. liquid paraffin for lambs, as specifics against *H. contortus*.

Le Roux (1932) has rightly criticised the nature of the infestation of the sample of adult animals selected as controls by these workers. Eight animals were selected, and six of these were infested with *H. contortus*, while two failed to show any infestation. Thus it can scarcely be claimed that this sample of animals fulfilled the requirements of the first fundamental assumption in the determination of the efficiency of an anthelmintic, viz. that all animals of a similar age and exposed to a similar risk of infestation are to be considered equally susceptible to infestation by the specific helminth.

The same objection cannot, however, be levelled against the lambs selected as controls. For lambs we find:

Table V. *The mean efficiency of tetrachlorethylene and carbon tetrachloride as specifics against* H. contortus *in lambs (Seddon and Ross)*

Treatment	*ps*	*E*	σ_E
Tetrachlorethylene 2·5 c.c.	12	0·2324	0·3327
Carbon tetrachloride 1 c.c. and liquid paraffin 4 c.c.	20	0·4591	0·3363

The comparison of the difference between efficiency of these two drugs as anthelmintics against *H. contortus* is not significant. Such a difference could be obtained between the means of two samples drawn from the same population about once in every sixteen trials.

Should it be believed that the investigations carried out by Seddon and Ross and by Roberts fulfil the requisite assumptions, then it is permissible to compare the results which these workers obtained for the same drug or mixture of drugs.

Believing this, we find, then, that in the hands of Roberts carbon tetrachloride 1 c.c. and liquid paraffin 4 c.c. is very significantly more efficient as a specific against *H. contortus* in lambs than in the hands of Seddon and Ross. Such a difference (viz. $0{\cdot}4591 - 0{\cdot}0923 = 0{\cdot}3668$) between the mean efficiency of the same mixture of drugs could only be obtained about once in a million trials under similar experimental conditions. Should, however, we compare the difference between the mean efficiency of tetrachlorethylene at a dose rate of 2·5 c.c. in the hands of these workers, we find that such a difference (viz. $0{\cdot}5972 - 0{\cdot}2324 = 0{\cdot}3648$) could occur by chance about 39 times out of every 100 similar trials.

These are two very good examples, demonstrating the danger of asserting a significance of a difference when only the absolute values of the means are known.

These last statements in which the results of different workers are compared, are not to be construed as being derogatory of the work of either investigators, but rather to show how necessary it is to be certain that all the experimental conditions are similar in both investigations, before an *ex cathedra* utterance should be made for or against any differences between the findings of workers.

SUMMARY

1. The assumptions underlying the current methods of determining the efficiency of drugs or a mixture of drugs as an anthelmintic are set out.

2. The method of assessing the efficiency of a given drug or mixture of drugs, as used by Seddon and Ross, is cited and objections to its value are given.

3. A method is suggested whereby the mean efficiency of a drug or mixture of drugs can be assessed, in terms of the initial and residual infestation of selected animals.

4. In the light of the suggested method, Roberts' data for the efficiency of various drugs and mixtures of drugs, as specifics against *H. contortus* infestation in sheep, are fully investigated.

ACKNOWLEDGMENTS

I am indebted to Prof. Harvey Sutton, Director of the School of Tropical Medicine and Public Health of the University of Sydney, for the continuance of the privilege of using the facilities of his biometric laboratory.

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ON CESTODES FROM BURMA

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(With 4 Figures in the Text)

The author wishes to express his sincere thanks to Dr Shen Tseng of Shantung, Tsingtao, China, for supplying diagnostic characters of three species of *Aploparaksis* Clerc 1903. All measurements are given in mm.

Family HYMENOLEPIDIDAE Fuhrmann 1907.

Subfamily Hymenolepidinae Perrier 1897.

APLOPARAKSIS Clerc 1903.

Aploparaksis kamayuta n.sp.

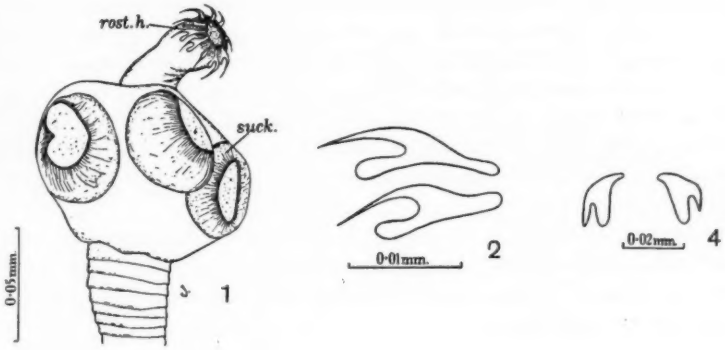
Host. *Capella stenura* Bonaparte 1830.

Locality. East Kamayut Ponds, Rangoon.

Maximum length 97·0, maximum breadth 0·95. Scolex 0·097 diameter. Rostellum 0·014–0·022 diameter. Rostellar hooks 10, 0·015–0·020 long. Suckers 0·027–0·049 in diameter. Genital pore at posterior half of the proglottis margin. The number of the inner longitudinal muscular bundles 33–45. Cirrus sac 0·102–0·175, usually 0·15–0·175 long, just reaching ventral longitudinal excretory vessel, in some cases just crossing it, extending less than one-fourth breadth of the segment. A well developed external vesicula seminalis present. Testis (0·1–0·115 in maximum diameter), approximately in centre of proglottis and in contact with its posterior border. Ovary nearly in centre of proglottis, anterior and partly ventral to testis, sometimes slightly poral. Receptaculum seminis fairly well developed. Uterus when young transversely elongated with saccular branches, which later become indistinct. The eggs globular or slightly ovoid; some of the eggs possess polar buds as mentioned by Tseng (1933), while the slit near the polar bud could not be seen. The longest diameter of outer shell 0·036–0·063 usually 0·052, and that of the inner shell, closely investing the onchosphere, 0·024–0·038.

The number of the rostellar hooks sufficiently separates from the present form *A. australis* Johnston 1911 and *A. dujardini* (Krabbe 1869): the size of the rostellar hooks, *A. crassirostris* (Krabbe 1869), *A. sinensis* Tseng 1933, *A. birulai* Linstow 1905, *A. furcigera* (Rudolphi 1819), *A. penetrans* (Clerc 1902), *A. murmanica* Baylis 1919, *A. hirsuta* (Krabbe 1882), and *A. veitchi* Baylis 1934; that of the cirrus sac *A. elisae* Skrjabin 1914, *A. australis*, *A. sinensis*, and *A. veitchi*; the number of the inner longitudinal muscular bundles

A. cirrosa (Krabbe 1869), *A. filum* (Goeze 1782), *A. larina* Fuhrmann 1921, *A. furcigera*, *A. murmanica* and *A. dujardini*; and the relative size of the cirrus sac *A. brachyphallos* (Krabbe 1869), *A. diminuens* Linstow 1905, *A. cirrosa*, *A. elisae*, *A. filum*, *A. fuliginosa*, *A. fusus* (Krabbe 1869), *A. hirsuta*, *A. sinensis* and *A. veitchi*. It is, therefore, necessary to create a new species for which I propose the name *Aploparaksis kamayuta* n.sp.[1]



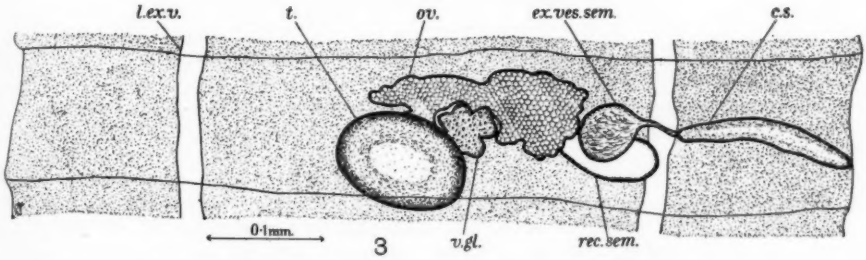


Fig. 1. Scolex of *Aploparaksis kamayuta* n.sp.
Fig. 2. Rostellar hooks of *Aploparaksis kamayuta* n.sp.
Fig. 3. Mature proglottis of *Aploparaksis kamayuta* n.sp.
Fig. 4. Rostellar hooks of *Hymenolepis bilharzii*.

Lettering for all figures: *c.s.* cirrus sac; *ex.ves.sem.* external vesicula seminalis; *l.ex.v.* longitudinal excretory vessel; *ov.* ovary; *rec. sem.* receptaculum seminis; *rost.h.* rostellar hook; *suck.* sucker; *t.* testis; *v.gl.* vitelline gland.

[1] 22. VI. 1935. After sending this paper to press, three new species have been added to the same genus, viz. *A. japonensis* Yamaguti, 1935, *A. scolopacis* Yamaguti, 1935 and *A. clerci* Yamaguti 1935. The differences in shape and size of the rostellar hooks of the first two, together with the disposition of cirrus sac and extension of ovary beyond the excretory vessels in the second, clearly separate them from the present species. *A. clerci*, which appears closely approaching the present form in sizes of rostellar hooks and cirrus sac, is easily distinguished for its specific disposition of ovary and testis besides other differences.

HYMENOLEPIS Weinland 1858.

Hymenolepis bilharzii (Krabbe 1869).

Host. *Corvus splendens insolens* Hume 1874.

Locality. West Kamayut, Rangoon.

Maximum length 60–120, maximum breadth 1·4. Scolex 0·18 long. Rostellum 0·03 diameter. Cirrus sac 0·096 long, not extending to ventral longitudinal excretory vessel. A small external vesicula seminalis present. Position of testes very variable, in some segments all three in the same straight line. In one segment only two testes, one poral and the other aporal; in another proglottis all three were aporal, the two posterior testes being near the hinder margin of the segment and the third anterior to them. Ovary greatly lobed and slightly poral in position.

The present form shows a great resemblance in the size and shape of the rostellar hooks, details of the egg and the size of the cirrus sac (with that described by Joyeux, Gendre, and Baer (1928)). Hence it is advisable to list it under the present species.

Hymenolepis globirostris Baer 1925.

Host. *Rattus rattus.*

Locality. University Estate, Rangoon.

A single tapeworm with its scolex so deeply buried in the inner wall of the duodenum that the rostellum was injured in detaching it. The body of the worm traversed the whole length of the intestine with the terminal portion continued in the rectum.

Length 196, breadth 1·4. Genital pore slightly posterior to the middle of the proglottis margin. Cirrus sac 0·248 in mature segments, but in gravid ones up to 0·267 × 0·032, nearly reaching longitudinal excretory vessel. Sometimes outer aporal testis slightly anterior to inner. Diameter of egg-shell 0·054. Diameter of onchosphere 0·035.

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SOME OBSERVATIONS ON THE BIONOMICS OF FLEAS IN EAST AFRICA

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The work detailed below was done by the writer at Nairobi in 1929, while serving as Medical Entomologist, Kenya.

Unless otherwise stated, the following are the conditions under which experiments were carried out:

Fleas were taken from wild hosts a few minutes before the beginning of an experiment; they were, therefore, of unknown age, but comparable in this respect with fleas leaving a dead rat. It is considered that they represent with fair accuracy the likelihood of survival of a "blocked" flea leaving a rat dead of plague. They were placed singly in test-tubes, $5 \times \frac{5}{8}$ in., plugged with cotton-wool, these test-tubes then being placed either in an incubator or cool chamber so as to maintain a constant temperature and relative humidity.

Experiments were started about 11.0 a.m. and examined daily at about 10.0 a.m., *i.e.* at intervals of approximately 24 hours; this tends to exaggerate the average length of survival, since a flea dying an hour after the experiment began would be considered to have lived 1 day. Similarly, in figures of egg-laying the notation 5/1 indicates that five eggs (which may have been laid at any time from 0 to 24 hours after the start of an experiment) were found after 1 day; the same notation applies (*mutatis mutandis*) to the other experiments. This notation differs from that used by Leeson (1932), who records a flea found dead after 4 days as having lived $3\frac{1}{2}$ days, so that the mean figures given in this paper are slightly higher than they would be if Leeson's notation had been employed.

While, for rapidity of completion of experiments, a technique not conducive to very long survival is desirable, the error introduced by examination at 24-hour intervals is proportionately large for such short lives; for this reason a standard technique should be one conducive to a moderately long life.

Fleas which had obviously been damaged in manipulation were not used. Eggs were left in the tube in which they were laid, food being added when larvae were observed to have hatched. The parent flea remained with the eggs unless still surviving when larvae hatched; in the latter event (which was not very frequent) she was transferred to a fresh tube. Larvae were transferred to a separate tube as soon as they were seen to have hatched, all the larvae from one batch of eggs being kept in one tube. Adult fleas reared were used for separate experiments on survival.

I. TEMPERATURES AND RELATIVE HUMIDITIES EMPLOYED

For experiments carried out at temperatures above the normal room temperature two incubators were available, for those below room temperature the only place which could be used was the outer chamber of the laboratory ice-plant. It was intended to carry out all the present series of experiments at the same relative humidity, and 100 per cent. was chosen; unfortunately relative humidity in the ice-plant was not within the writer's control, so the experiments at lower temperatures had to be carried out at a different relative humidity. Both factors were checked daily by means of thermometers and a hygrometer. The nominal temperatures and relative humidities used are discussed below.

In subsequent sections of the paper the relative humidities are often omitted for the sake of brevity; a mention of the temperature of 30° C. (*e.g.*) implies a relative humidity of 100 per cent.

(*a*) 15° C. *and relative humidity* 60 *per cent.* The mean temperature in the outer chamber of the ice-plant varied between 14·8 and 15·0° C., with an average of 14·9° C., the maximum temperature was 16° C. and the minimum 14° C. The mean relative humidity varied between 58·0 and 58·7 per cent., with an average of 58·4 per cent.; the maximum recorded was 65 and the minimum 54.

(*b*) 20° C. *and relative humidity* 100 *per cent.* In this and the next set of conditions the high relative humidity was obtained by placing a shallow dish of water in the incubator. In view of the known unreliability of hygrometers at high relative humidities it is probable that the relative humidity in both sets of experiments approximated to 100 per cent.

The mean temperature in the incubator varied between 20·5 and 22·7° C., with an average of 21·6° C.; the maximum recorded was 24 and the minimum 19. Mean relative humidity varied between 94·4 and 94·6 per cent., with an average of 94·5 per cent.; the maximum was 96 and the minimum 92 per cent.

(*c*) 30° C. *and relative humidity* 100 *per cent.* The mean temperature varied between 28·3 and 30·0° C., with an average of 29·2° C.; the maximum recorded was 31° C.

Mean relative humidity varied between 95·5 and 96·9 per cent., with an average of 96·0 per cent.; the maximum was 99 per cent. and the minimum 92 per cent.

II. SURVIVAL OF UNFED FLEAS

(*a*) *With and without "perch"*

A preliminary experiment was carried out to see if the use of a "perch" (a small piece of cloth introduced into the tube) would prolong life and conduce to egg-laying. The experiment was carried out at 20° C. and relative humidity 100 per cent., with fleas from captured rats.

Without the "perch" the average life of thirty-four males of *Xenopsylla brasiliensis* was 7·1 days, and of thirty-seven females was 12·6 days. With the "perch" the average life of eighteen males and twelve females of this species was 7·2 and 10·5 days respectively.

Similar figures were obtained with *X. cheopis*. The average life of thirty-six males without "perch" was 5·6 days and of twenty-two females was 9·9 days; with "perch" the figures were 6·2 days for forty males and 7·1 days for thirty-two females.

With regard to egg-laying, 40 per cent. of the females of *brasiliensis* and 82 per cent. of the females of *cheopis* without a "perch" laid eggs, and the number of eggs per laying female was 5·2 and 4·6 respectively for the two species. With a "perch", 42 per cent. of *brasiliensis* females and 59 per cent. of *cheopis* laid eggs, and the average production per laying female was 3·8 eggs for each species.

The figures are obviously insufficient and the differences cannot be considered significant, but it is interesting to note that, while the length of life of males is practically the same with and without "perch", the presence of the "perch" appears definitely unfavourable to females. In the case of both species the length of life, percentage of laying females, and the number of eggs per laying female is less with a "perch" than without one. The last item is unlikely to be affected by length of survival since practically all the eggs in all experiments were laid on the first day (p. 485).

As this experiment showed no significant difference in favour of the use of a "perch", all subsequent experiments were done without one.

(*b*) *Survival of newly hatched fleas*

The fleas used in this experiment were those which hatched in the course of the breeding experiments detailed below. As only small numbers of fleas were available, the experiments were performed under only one set of conditions, a nominal temperature of 20° C. and relative humidity of 100 per cent. The results are shown in Table I.

Table I. *Length of survival, in days, of unfed newly hatched fleas at a nominal temperature of* 20° C. *and relative humidity of* 100 *per cent.*

Sex	No. used	Minimum life	Maximum life	Mean life and standard error of mean*	Standard deviation
			Xenopsylla brasiliensis.		
Male	26	8	46	21·0±1·756	8·9533
Female	22	8	44	21·1±2·046	9·5982
			Xenopsylla cheopis.		
Male	17	8	36	21·6±2·140	8·8251
Female	18	5	31	21·7±1·627	6·9003

* Thanks are due to Mr C. G. Hansford, who kindly worked out standard error and standard deviation in this and subsequent tables.

The different sexes, in the case of both the species of *Xenopsylla* used, show no significant difference in length of survival, the average expectation of life being 20·9 days for males and 20·7 days for females of *X. brasiliensis*, and 21·6 and 21·7 days respectively for the sexes of *X. cheopis*.

Leeson's figures, which are most nearly comparable with those given here, are those for *cheopis* at a temperature of 18° C. and relative humidity 90 per cent. The average survival of his fleas under these conditions was 16·5 days in the case of males and 12·5 in the case of females. Bearing in mind that the number of fleas employed (forty-nine males and forty-seven females in the case of Leeson and seventeen males and eighteen females in the present work) is small and that the conditions were not exactly similar, it is still very interesting to note that the average survival in the present experiments is considerably higher than in Leeson's work, in spite of the fact that the maximum survival of males in the present experiments is much lower than in Leeson's work and that of females not much greater. This becomes the more significant when it is noted that the temperature and relative humidity in the present work were both slightly above those used by Leeson, since at 23° C. and relative humidity 95 per cent. the mean survival of his fleas was 6·4 days in the case of females, or approximately half those at 18° C. and relative humidity 90 per cent., and slightly lower than those at 23° C. and relative humidity 90 per cent.

The difference is not accounted for by the difference in notation. Since all Leeson's fleas were recorded as having lived half a day less than those found dead on the same day of the experiment in the present work, the average length of life of his fleas should be 0·5 day less than that of the writer's fleas; actually the difference is very much greater. The present figures, small as they are, fully confirm Leeson's finding that the expectation of life in unfed newly hatched fleas is the same for both sexes. The importance of this point becomes obvious when survival of wild-caught fleas is discussed.

(*c*) *Survival of wild-caught fleas*

The conditions of this series of experiments have been detailed on p. 480 and the temperatures and relative humidities on p. 481. The results are shown in Table II. The most obvious fact which emerges is the difference in expectation of life between the sexes. This is, as shown above, quite contrary to findings with unfed newly hatched fleas, but it is consistent under all the sets of conditions used and for all species except *Dinopsyllus lypusus*, of which the numbers used were very small. It is not due to a greater proportion of males being damaged in manipulation, since the majority of such fleas would be found dead after the first day and the proportion of males surviving only 1 day is little, if any, higher than that of females. As noted previously, the age of such fleas at the beginning of an experiment is unknown, but they were taken from many different rats and care was always taken to distribute fleas from any one rat instead of using all of them under one set of conditions.

They constitute, therefore, a random sampling of the rat-flea population, and it is believed that differences shown by the figures are probably significant in cases where the number of fleas used is sufficient. (Mr C. G. Hansford kindly informs the writer that the differences are statistically significant except in the cases of *X. cheopis* at 20° C. and of *D. lypusus*.)

Experiments with *X. brasiliensis* were performed under all three sets of conditions, but the number used at 30° C. was so small that the results could have no significance and are omitted. It is quite clear that a temperature of 15° C. and relative humidity of 60 per cent. is less favourable to survival for both sexes of this species than 20° C. and relative humidity 100 per cent.

In the case of *X. cheopis*, the numbers used at 15° C. are very small, but

Table II. *Length of survival unfed of wild-caught fleas, in days*

Temp. (° C.) and relative humidity (%)	Sex	No. used	Minimum life	Maximum life	Mean life and standard error of mean	Standard deviation
			Xenopsylla brasiliensis.			
15, 60	M.	27	1	10	4·6 ±0·520	2·7009
	F.	31	1	14	8·1 ±0·667	3·7143
20, 100	M.	55	1	17	6·85±0·470	3·4823
	F.	55	1	28	11·3 ±0·686	5·0884
			Xenopsylla cheopis.			
15, 60	M.	17	1	7	2·8 ±0·345	1·4235
	F.	25	1	12	5·1 ±0·615	3·0811
20, 100	M.	181	1	*16	5·5 ±0·234	3·1420
	F.	163	1	21	7·15±0·322	4·1076
30, 100	M.	113	1	13	3·2 ±0·213	2·2605
	F.	145	1	13	4·9 ±0·245	2·9506
			Leptopsylla segnis.			
20, 100	M.	14	1	6	2·4 ±0·343	1·2839
	F.	32	2	9	4·9 ±0·393	2·2252
			Dinopsyllus lypusus.			
20, 100	M.	19	1	14	8·0 ±0·713	3·1092
	F.	20	1	24	8·05±1·564	6·9923

* A single record of survival for 28 days is probably erroneous.

they agree with those for *brasiliensis* in indicating that this set of conditions is unfavourable to survival. A moderately large number of experiments were done at 30° C.; here, also, it is quite clear that conditions were less favourable than at 20° C., although in this case the relative humidity was the same at both temperatures.

III. Oviposition

The particulars of eggs laid by female fleas in the course of the experiments are shown in Table III. The random error in these figures is undoubtedly large, as it was a very common experience to find that all the fleas of a given batch laid eggs while of the next batch none did so. For this reason the figures can only be accepted as probably significant if confirmed by the case of another species of flea. This is the case with the two lower temperatures,

although the number of fleas used at 15° C. is very low. The very exact correspondence between numbers of eggs per female of *brasiliensis* and *cheopis* at 15 and 20° C. is probably accidental, but there is a very strong suggestion that 15° C. and relative humidity 60 per cent. is unsuitable to egg-laying. There is also an unconfirmed suggestion that 30° C. and relative humidity 100 per cent. is unsuitable, and all these differences are found by Mr Hansford to be statistically significant.

These suggestions are not accounted for by the fact that the average life of fleas was greater at 20° C. Of the 751 eggs laid during the experiments, 716 were laid on the first day. Those laid on other days were as follows: *X. brasiliensis* at 15° C., 11/2, four batches; *X. cheopis* at same temperature, 3/3, by one female; *X. brasiliensis* at 20°, 16/2, 1/3, four batches; *X. cheopis* at same temperature, 1/2, 1/3; *Leptopsylla segnis* at same temperature, 2/2 by

Table III. *Particulars of eggs laid by female wild-caught fleas kept at different temperatures and relative humidities*

Temp. (° C.) and relative humidity (%)	No. of females used	No. which laid eggs	% which laid eggs	Total eggs laid	Average eggs per laying female	Average eggs per female	Standard deviation (for eggs per female)	Standard error of mean (for eggs per female)
			Xenopsylla brasiliensis.					
15, 60	31	9	29	19	2·1	0·6	1·20 ±	0·22*
20, 100	55	32	58	136	4·2	2·8	3·36 ±	0·45*
			Xenopsylla cheopis.					
15, 60	25	5	20	16	5·0	0·6	1·47 ±	0·29*
20, 100	156	84	54	376	4·5	2·4	2·73 ±	0·22*
30, 100	145	43	30	150	3·5	1·0	1·89 ±	0·16*
			Dinopsyllus lypusus.					
20, 100	20	3	15	14	4·6	0·7	1·69 ±	0·38
			Leptopsylla segnis.					
20, 100	32	12	38	40	3·3	1·3	2·49 ±	0·44

* Difference significant ($P < 0{\cdot}01$).

one female. The four batches of *brasiliensis* at 15° C. were laid by females which had not laid eggs earlier; all the other batches were from females which had laid on the first day. At 30° C., all the eggs were laid on the first day. The facts indicate a probability that egg-laying is delayed to some extent at 15° C. and thus confirm the previous suggestion that this temperature is unsuitable for oviposition. The delay in laying is much below the average life of the female flea at this temperature and (as previously mentioned) cannot account for the small number of eggs.

Except in cases where only a small number of batches were obtained, the minimum batch of eggs was always one; the maximum batches were as follows: *X. brasiliensis* at 15° C. five, at 20° C. twelve; *X. cheopis* at 15° C. five, at 20° C. ten, at 30° C. seven; *D. lypusus* at 20° C. five; *L. segnis* at 20° C. thirteen.

IV. Hatching of eggs

Since the treatment of eggs and larvae was uniform throughout and the age of both was known with accuracy, the observations are more exact than in the case of the wild-caught fleas.

The results are shown in Table IV. A temperature of 15° C. with relative humidity 60 per cent. is obviously entirely unsuited to hatching of eggs of both species of *Xenopsylla*, and there is a very strong indication that 30° C. and relative humidity 100 per cent. is also unsuitable in the case of *cheopis* (sufficient eggs of *brasiliensis* laid at this temperature were not available). The difference in the percentage of eggs of *brasiliensis* and of *cheopis* which hatched at 20° C. is perhaps not significant, but suggests that these conditions are

Table IV. *Particulars of hatching of eggs of various species of fleas at different temperatures and relative humidities. Hatching periods are given in days*

Temp. (° C.) and relative humidity (%)	No. of eggs used	No. of eggs which hatched	% of eggs which hatched	Mean egg period and standard error of mean	Minimum egg period	Maximum egg period	Standard deviation
			Xenopsylla brasiliensis.				
15, 60	78	0	0	—	—	—	—
20, 100	191	119	62·3	9·4 ±0·117	7	14	1·2716
			Xenopsylla cheopis.				
15, 60	37	*1	—	—	—	—	—
20, 100	371	176	47·4	8·3 ±0·063	6	13	0·8391
30, 100	147	20	13·6	6·35±0·318	5	9	1·4244
			Leptosylla segnis.				
20, 100	36	22	61·1	6·4 ±0·204	5	8	0·9534

* After 11 days.

more suited to the former than to the latter. Under these conditions there is considerable uniformity as regards the length of the egg period, the figures of distribution showing that the great majority of eggs hatch after 7, 8 or 9 days in the case of *cheopis* and 8, 9 or 10 days in *brasiliensis*; the difference in the average egg period of the two species thus appears to be perfectly genuine. At 30° C. the average egg period of *cheopis* is shortened by nearly 2 days, but the number which hatched is small.

V. Length of larval and pupal period

The larvae used in these experiments were those obtained in the hatching experiments just described. They were always kept at the same temperature and relative humidity at which the eggs had been laid. In the earlier experiments the food used was bran plus a pinch of dried ox-blood; in the later experiments maize-meal was substituted for the bran.

In order to get a little closer to natural conditions, the food was not sterilised. In the confined space of the test-tubes moulds created much worse conditions than would be common in nature, while the larvae of a moth (*Ephestia* sp.) spun together the unsterilised food and thus not only created difficulties in manipulation, but also made bad breeding conditions for the flea larvae. For this reason little importance can be attached to the percentage of adults which emerged, but the figures for the length of larval and pupal life are believed to be entirely reliable.

To induce hatching in adult fleas resting within the cocoon, the test-tube was gently tapped and this was a standard part of the technique after the 14th day of each experiment.

Table V. *Emergence of adult fleas from larvae less than* 24 *hours old, at* 20° C. *and relative humidity* 100 *per cent.*

No. of larvae used	No. of adults which emerged	Percentage which emerged	Sex	Mean larval and pupal period and standard error of mean	Minimum larval and pupal period	Maximum larval and pupal period	Standard deviation
			Xenopsylla brasiliensis.				
119	52	43·7	Male	55·5 ± 2·993	46	69	5·2802
			Female	47·4 ± 1·218	41	63	5·7126
			Xenopsylla cheopis.				
176	47	26·7	Male	50·7 ± 1·150	40	67	6·0862
			Female	45·5 ± 1·503	38	58	6·5521

In order to avoid congestion in the limited space available, all breeding experiments were ended after 3 months and the survivors (if any) were thrown out. As the longest period for larval and pupal stages which was obtained in these experiments was 69 days, it is believed that no error was introduced.

No attempt was made to separate the larval and pupal periods for fear of injuring the insect.

The results are shown in Table V, but the single larva of *cheopis* which hatched at 15° C. and the twenty larvae of the same species which hatched at 30° C. did not produce adults and they are omitted from the table. Of twenty-two larvae of *L. segnis* kept at 20° C. nearly all died of the effects of mould, a female adult being bred after 40 days and two males after 41 and 44 days respectively; these also are omitted from the table.

The most striking fact shown by the table is that in both the species of *Xenopsylla* employed the female has a shorter larval and pupal period than the male. This is shown more clearly in the original figures: in the case of nine batches of *cheopis* which produced both sexes in only two instances did a female emerge after a male of the same batch, while in six batches of *brasiliensis* of mixed sex this never occurred.

VI. Summary

Observations have been made on the survival of unfed rat-fleas, both newly hatched and wild-caught, under controlled conditions of temperature and relative humidity, and further observations on oviposition, hatching of eggs and emergence of adults under the same conditions.

The results show that, of the three sets of conditions employed, a temperature of approximately 20° C. and relative humidity of about 100 per cent. are the most favourable for survival of adults, hatching of eggs and production of a further generation of adults. The two other sets of conditions (temperatures of 15 and 30° C., with relative humidities of 60 and 100 per cent. respectively) are entirely unsuitable for the breeding of the species of fleas used.

At 20° C. and relative humidity 100 per cent. the whole pre-adult life of *Xenopsylla brasiliensis* and *X. cheopis* is, on the average, approximately 8–9 weeks, the minimum being 6 weeks. The female in both species is shown to have a distinctly shorter larval and pupal life than the male.

REFERENCE

Leeson, H. S. (1932). The effect of temperature and humidity upon the survival of certain unfed rat fleas. *Parasitology*, **24**, 196.

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IXODES RICINUS IN RELATION TO ITS PHYSICAL ENVIRONMENT

III. CLIMATE AND REPRODUCTION

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(With 2 Figures in the Text)

CONTENTS

	PAGE
I. Pre-oviposition period	489
(*a*) Duration of pre-oviposition period at different temperatures and humidities	489
(*b*) Lower limiting temperatures for maturation of ovaries	491
II. Oviposition	491
(*a*) Limiting temperatures and humidities	491
(*b*) Influence of temperature and humidity on course of oviposition	491
III. Development of the egg	493
(*a*) Limits of temperature and humidity	494
(*b*) The threshold of development	495
(*c*) Influence of oviposition-conditions on development and fertility of eggs	497
(*d*) Velocity of development of eggs at different temperatures	498
IV. Discussion	499
References	500

That part of the physiological life history of the tick, *Ixodes ricinus*, which is dealt with in the present paper, falls into three distinct phases: maturation of the gonads, oviposition, and development of the egg; these three are collectively referred to as "reproduction".

I. Pre-oviposition period

(*a*) *Duration of pre-oviposition period at different temperatures and humidities*

The influence of the different climatic factors on the development of the gonads of insects and arachnids has received little attention. In the case of ticks, Cunliffe (1921) showed that the pre-oviposition period in *Ornithodorus moubata* averaged 29 days at 22° C. and 15 days at 30° C. Bishopp (1932) found it to be longer, in the cattle tick, at low than at high temperatures. Observations on the duration of the period at different seasons have been recorded by Hooker, Bishopp and Wood (1912) and Bishopp and Wood (1913) for many of the North American ticks. No data have been recorded of the

effect of climate on this period in *Ixodes ricinus*. The existing information on the duration of the period may be summarised as follows:

Authors	Temperature ° C.	Days
Wheler (1899)	—	22
Samson (1909)	—	14
Nuttall (1913)	24	17–24
Olenev (1927)	—	4–24
Elmanov (1930)	—	6–14
Falke (1931)	20	8–10
MacLeod (1932)	15–21	14–52 (usually 15–22)

Newly gorged female ticks, each with a male attached, were subjected to different combinations of temperature and humidity as the ticks became available. Table I records the time elapsing before oviposition began.

Table I. *Duration of the pre-oviposition period in days at different temperatures and humidities*

	Temp. ° C.									
R.H.	2–3	10	14–16	17–19	20	22·5	25	27·5	30	33–34
100	No egg-laying	.	.	20	.	12	.	32		.
		33	15	16	16	10	22	25	+	+
	.	.	.	13	.	17	9	16	+	+
	.	.	19	21	20	11	16	11	+	+
	.	.	.	21	.	8	8	9	+	.
	.	.	.	14	.	.	.	.	.	.
95	.	.	16	.	.	8	10	.	.	.
	.	.	.	.	.	.	10	.	.	.
90	.	.	.	.	.	.	20	.	.	.
	.	.	.	.	.	.	11	24	+	.
	+	.	15	.	16	.	11	11	+	+
	.	.	.	.	.	.	10	.	.	.
	.	.	.	.	.	.	9	.	.	.
85	.	.	24	.	.	16	10	.	.	.
80	.	31	17	.	25	.	12	.	.	.
	.	.	.	.	.	.	10	.	+	+
	.	.	.	.	.	.	+	.	.	.
75	.	.	25	.	+	.	+	.	.	.
	.	.	+	.	+	.	.	.	.	.

+ indicates death without oviposition.

At any particular combination of temperature and humidity, there is considerable variation in the duration of the pre-oviposition period. The apparent tendency for the period to be shorter at high temperatures than at low is rendered insignificant by this irregularity; all that can be safely inferred from the table is that at the extreme limits of temperature and humidity, *i.e.* at 27·5° C., 100–90 R.H., 20° C., 80 R.H., and 14·16° C., 75 R.H., the period is longer than at median temperatures and high humidities. At many of the extremes, particularly of humidity, oviposition was abortive, only some few dozen eggs being laid.

It is of interest that the shortest period obtained at any combination was 8 days, whereas two Russian workers, Olenev and Elmanov, record shorter periods.

(*b*) *Lower limiting temperatures for maturation of ovaries*

In Table I, "no egg-laying" is recorded for the combination 2–3° C., 100 R.H. This means that in the two-months' period during which the females were under observation, oviposition had not commenced. The experiments were then discontinued. It was apparent, however, that the method used involved a possible source of error. The absence of oviposition might not necessarily mean that the gonads were not mature; it might merely be due to the two processes having different critical temperatures. To test this, a gorged female which had been kept at room temperature, 15–18° C., for 7 days, was placed at 2° C. and left for 6 days. It was then returned to room temperature, 100 R.H., and oviposition started 6 days later, *i.e.* 19 days after engorgement. Another female, immediately after engorgement, was placed at 2° C., 100 R.H., and left there for 3 months. Nine days after removal to room temperature, egg-laying commenced. The experiment was repeated, two females being left for 3 months at 2·3° C. Oviposition began 10 days after removal to room temperature. It would therefore appear, in view of the normal duration of the period at room temperature, that some degree of development of the ovaries took place at the lower temperature, *i.e.* that maturation may occur to some extent at temperatures as low as 2–3° C.

II. OVIPOSITION

(*a*) *Limiting temperatures and humidities*

A study of Table I indicates the limiting conditions of temperature and humidity for oviposition. A striking feature of the result is the comparatively low upper limit of temperature—between 27·5 and 30° C. Again, as in metamorphosis of gorged ticks and also in survival of unfed ticks, the lower limiting humidity is high—75–80 R.H. at temperatures of 14–25° C.

As has been stated above, no oviposition took place at 2–3° C. over periods of 3 months. To determine whether this was due to the inhibitory effect of such a low temperature, or merely to the extremely slow rate of maturation of the eggs, the following experiment was made.

Three females which had commenced egg-laying at room temperature were placed at 2–3° C. Oviposition ceased, and no eggs were laid during the next three months. The females were then returned to room temperature, and within a few days oviposition recommenced. It would appear, therefore, that whereas development of the ovaries may not be inhibited at this temperature, the actual process of oviposition is inhibited.

(*b*) *Influence of temperature and humidity on course of oviposition*

No exact evidence is recorded of the effect of temperature or humidity on oviposition in this tick, or, indeed, any information with regard to the factors

influencing the process. Nuttall (1913) records that oviposition lasted 32–45 days in May, and 44–45 days in April.

The following records refer to female ticks exposed to controlled conditions at different times, as opportunity arose. Table II records the total number of eggs laid by females in saturated air, and at different temperatures, together with the time taken. As might be expected, there is a considerable variation in the total for any one temperature, since the amount and nature of the blood meal will naturally affect the total. It is apparent from the table that the average total is not significantly affected by temperature. This is in agreement with the results obtained by Hunter and Hooker (1907) for *Margaropus annulatus*. Cunliffe (1921), however, found that, in the case of *Ornithodorus moubata*, the egg total at 30° C. was 50 per cent. higher than that at 22° C.

The average time taken for completion of oviposition is progressively less with increasing temperatures. Although the results, taken individually, are

Table II. *Number of eggs laid at different temperatures and in saturated air*

Temp. ° C.						Mean total	Mean time	Mean daily average
14–18	Total output	1069	2156	1376				
	Days	40	33	36	—	1534	36	44
	Daily average	27	68	38				
22·5	Total output	2052	1789	2263	1890			
	Days	32	28	34	23	1998	29	69
	Daily average	64	67	64	82			
25	Total output	1890	2400	1043	1289			
	Days	26	25	18	20	1656	22	73
	Daily average	73	96	58	65			
27·5	Total output	832	2300					
	Days	19	22	—	—	1566	20	77
	Daily average	49	105					

not significantly different from each other, the regular trend of the averages suggests that there is a correlation between time and temperature (compare Nuttall's finding, quoted above). The average number of eggs per day has been estimated in each case, and the means of the results (end column) show a progressive increase with higher temperatures, suggesting that the decrease in the time taken at higher temperatures is due to the accelerated rate of oviposition, and not to a decrease in the number of eggs laid.

Table III gives the egg totals, time taken, and daily average at 25° C. and relative humidities of 100, 90 and 80. Here, also, the results, though not statistically significant, are suggestively uniform in the trend of their means, and might justifiably be interpreted as indicating that the progressive decrease in the daily average, with decreasing humidities, is due, not to a lengthening of the time taken for oviposition, since the time is more or less constant (column 2 of averages), but to a decrease of the total (column 1). In other words, a decrease of temperature slows down the rate of oviposition, whereas a decrease in humidity results in a reduction of the total number of eggs laid.

Table III. *Number of eggs laid at different humidities and* 25° C.

R.H.						Mean total	Mean time	Mean daily average
100	Total output	1890	2400	1043	1289			
	Days	26	25	18	20	1656	22	73
	Daily average	73	96	58	65			
90	Total output	813	1840	980				
	Days	15	21	19	—	1211	18	65
	Daily average	54	88	52				
80	Total output	1120	1310	727	948			
	Days	24	18	18	19	1026	19	53
	Daily average	47	73	40	50			

Fig. 1 illustrates the course of oviposition, A, at one humidity and two temperatures; B, at one temperature and two humidities. Each curve is based on the actual daily figures for one female. Daily counts were made for all the females; the curves were drawn for each and are more or less similar to the types presented. These have been chosen merely because they approximated most closely to the average times and totals for their particular conditions. They represent, respectively, the following ovipositions:

Conditions	Total	Days
14–18° C. 100 R.H.	1376	36
25° C. 100 R.H.	1289	20
25° C. 80 R.H.	948	19

It will be seen that the oviposition curve betrays a uniform character, the daily deposition of eggs rapidly rising to its maximum, then falling away, more or less slowly, as the ovary becomes exhausted. Under favourable humidity conditions, the peak of the curve is higher, and is reached more rapidly, at the higher temperature, and exhaustion occurs after a shorter period. At the lower temperature, where metabolic activity is slower, the ovary takes longer to reach its maximum functioning rate, and maintains it over a longer period; the curves are, however, essentially similar. In the case of the female at the unfavourable humidity of 80 R.H. the result is different. At first, its oviposition curve agrees with that of the female at 100 R.H., but, within a few days, under the adverse moisture conditions, the ovary fails to maintain the daily output, which rapidly drops to a low value, and thereafter dwindles to zero. Oviposition ceases, not because the ovary has exhausted its potentiality for egg production, but because of the adverse external conditions.

III. DEVELOPMENT OF THE EGG

It would appear that the only controlled experiments on the effect of climate on the development of the egg of *Ixodes ricinus* are those of Olenev (1924, 1927). He states (1924) that, whereas under natural conditions eggs took 8 weeks to hatch, under artificial conditions at a temperature of 25–30° C., they hatched in 3 weeks. They were killed by a humidity of 50–80 R.H. In a later paper (1927) he describes an experiment in which he placed eggs (1) on

ice (0–17° C.), (2) in corridor (1–12° C.), (3) in forest (10–20° C.), (4) in tent (15–20° C.) and (5) in kitchen (25–30° C.). Hatching occurred after 300–400 days, 140 and 67 days, 61, 44 and 25 days respectively.

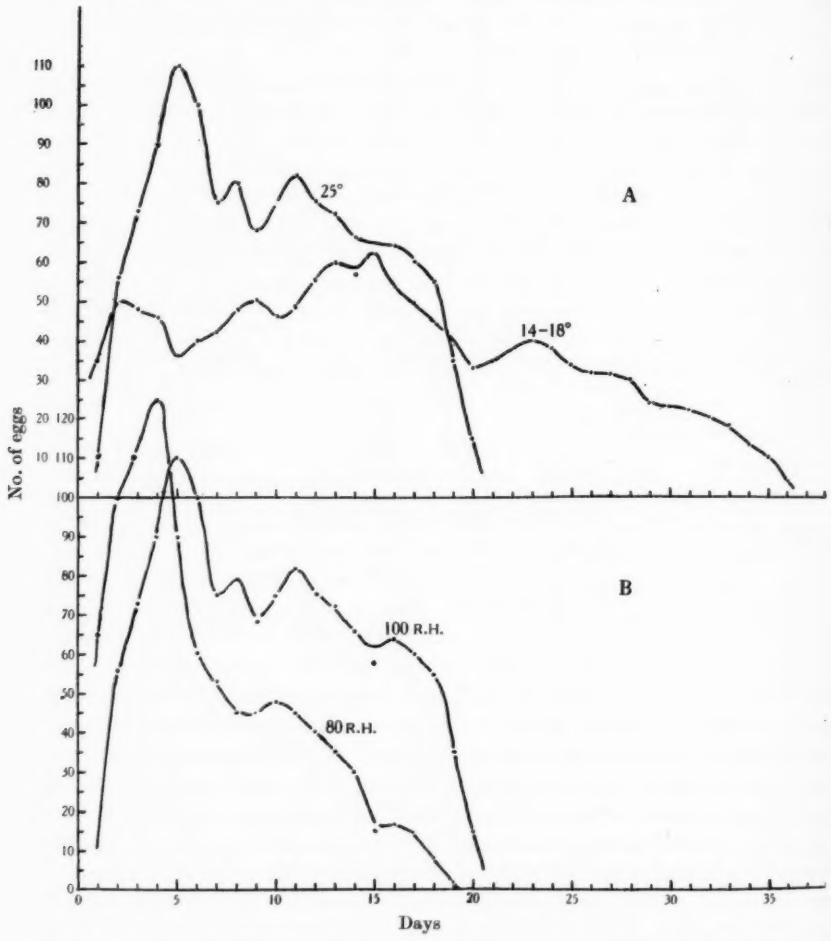

Fig. 1. The course of oviposition: A at 100 R.H. and temperatures of 14–18 and 25° C.; B at 25° C. and R.H. of 100 and 80.

(a) *Limits of temperature and humidity*

It was hoped to obtain information both on the limiting conditions and on the percentage viability of eggs under these conditions by subjecting batches of eggs of known number to the various combinations of temperature and humidity. Several such experiments were set up, and in all of them the same

result was encountered—the eggs shrivelled, and no hatching occurred except at one or two combinations, notably at 25° C. and the higher humidities. It would seem that the manipulation of the eggs, incident on their enumeration adversely affects their viability to a very marked extent, so that, even under favourable conditions, there is a tendency for them to shrivel. The expedient of using complete egg clusters was then resorted to; the results were recorded merely as "hatching" if any larvae appeared, or "death", no account being taken of the differential mortality rates. Table IV records the results of the various experiments. The values refer to the number of days between exposure of the egg cluster to the particular conditions and the hatching of the first larvae. A plus sign indicates that no eggs hatched.

It will be seen that there is a wide variation in the times recorded, even for one combination of temperature and humidity. This is doubtless due to

Table IV. *Time in days taken for development of eggs*

R.H.	Temp. (° C.) 14–18	15–22	17–23	21–23	25–26	27·5	30	35
100	72	50	37	32	*24	*20	*18	+
	67	43	37	*30	23	16	+	+
	+	.	+	30	22	15	+	.
	+	.	+	22	+	.	.	.
	.	.	.	+	+	.	.	.
	.	.	.	.	+	.	.	.
90	+	.	37	28	27	19	19	.
	+	.	37	25	25	18	+	.
	.	.	.	+	24	17	+	.
	.	.	.	.	+	+	.	.
80	.	.	44	35	+	22	+	.
	.	.	39	+	+	+	+	.
	.	.	+	.	.	.	+	.
75	+	.	.	+	+	+	.	.
	.	.	.	.	+	.	.	.

+ = death of egg.

such variables as the age of the eggs, which varied from 0 to 10 days, and the temperature of the individual thermostats which differed slightly for the different experiments which were carried out over a period of two years. Four results are, however, strictly comparable; these are indicated by asterisks and will be referred to later (p. 498).

It would appear that the lower limiting humidity for development of the egg corresponds with that for development of the later stages, being in the neighbourhood of 80 per cent. saturation. The upper temperature limit for viability of eggs seems to be slightly lower than that for the gorged larvae and nymphs, apparently lying between 30 and 35° C.

(*b*) *The threshold of development*

It has been shown in a previous paper that the Thermal Constant theory cannot be used as a basis for the estimation of the threshold of development of the tick. The approximate threshold might be determined by the method

of plotting the velocity curve of development and producing it below the lowest point experimentally obtainable. This method cannot be used here since sufficiently exact information on the developmental velocity of the egg at different temperatures is not available. It became necessary, therefore, to devise an alternative method.

This consisted in exposing eggs to two temperatures sufficiently close to each other to obviate the possibility of their requiring, for completion of development, different amounts of heat—expressed in effective day-degrees or day-degrees over the threshold. The result allows the probable threshold to be obtained by calculation. It was felt desirable, in order that a true result might be obtained, to reproduce, as far as possible, the conditions under which eggs would develop in nature, *i.e.* saturated air and a fluctuating temperature. Clusters of over two hundred eggs of the same age were used; the date of commencement of hatching in each case may reasonably be taken, therefore, as reflecting fairly accurately

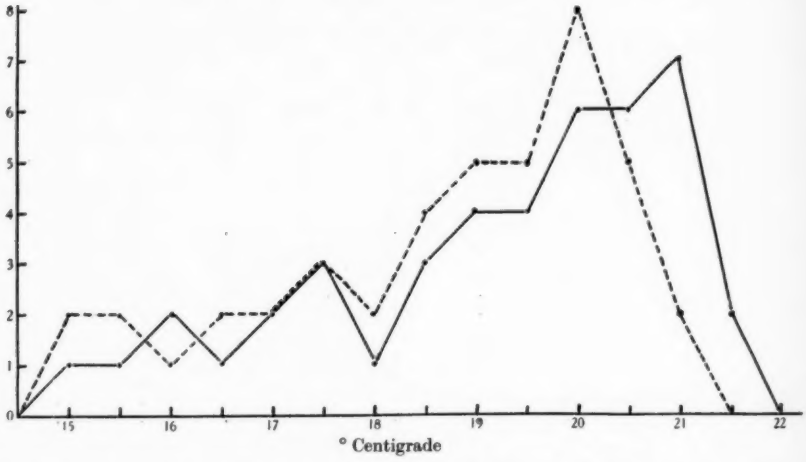

Fig. 2. The relation between the temperature of a water-bath (whole line) and an evaporation-cooled system (interrupted line). The curves show the frequency-distribution of temperatures within their ranges of fluctuation.

the true shortest time required for development under the particular conditions obtaining, since with such numbers individual variation is unlikely to affect the result. The egg clusters, which had not been counted or otherwise handled were exposed in tubes over water in closed humidity systems. A temperature range exhibiting moderate fluctuations was obtained by the use of a water-bath with a cold-water circulation. The difficulty lay in reproducing these conditions with a slightly higher temperature, *i.e.* in obtaining a different temperature range, the fluctuations of which should correspond in frequency and magnitude to those of the water-bath. An evaporation-cooled system was found to fulfil these requirements. A humidity system was wrapped in cloth, which was kept moist by a steady trickle of water. The rate of water-flow in both systems was adjusted so that their temperatures differed by only a half-degree centigrade. Daily temperature records were then taken, and it was found that the temperature of the evaporation-cooled system maintained a fairly consistent relation to that of the water-bath. Fig. 2 shows the frequency-distribution curves of temperature of the two systems from the commencement of the experiment

until commencement of hatching in the water-bath. It will be seen that there was a close quantitative correlation between the fluctuations of the two systems, the evaporation-cooled system remaining approximately a half-degree cooler than the water-bath.

The experiment was initiated on the same day as that on which the eggs were laid. Hatching commenced in the water-bath on the 43rd day, in the evaporation-cooled system on the 50th day.

The mean temperature of the former during the 43 days was 19·2° C., that of the latter, during the 50 days, 18·7° C.

Therefore, the total heat increment in day-degrees in each case was $19{\cdot}2 \times 43$, or 826, and $18{\cdot}7 \times 50$, or 935.

Let x = the threshold of development; then, $826 - 43x$ = the number of effective day-degrees = $935 - 50x$ (since it is assumed that the necessary number of effective day-degrees is the same for two temperatures so close together). Therefore $x = 15{\cdot}5$.

This result confirms that obtained in the case of the gorged ticks, *i.e.* that the threshold of development lies in the neighbourhood of 15° C.

This conclusion is, of course, permissible only if it be assumed that hatching of the egg occurs immediately development of the contained larva is completed, and is not dependent on the occurrence of a particular set of extrinsic conditions. This assumption seems reasonable, but there is no direct evidence on this point. Incidentally, it has been found that hatching of fully developed larvae would not occur at 2–3° C. over a period of exposure of 48 days.

(c) *Influence of oviposition-conditions on development and fertility of eggs*

It must be borne in mind that the development of the egg presents rather a different problem from that of the gorged larva or nymph, since its development represents but one phase, prior to the inception of which the egg, or rather the ovigerous female, is exposed to variable climatic vicissitudes. It is, therefore, necessary to consider any influence which such variations may have on the future history of the egg.

In the following experiment, the conditions considered were: low temperature with low humidity, low temperature with high humidity, high temperature with high humidity, and high temperature with low humidity. The effect of each of these combinations of conditions during development was tested on eggs which had been laid under each of these combinations of conditions. Gorged females which had begun egg-laying were exposed to the appropriate conditions for one week. The egg clusters laid during this period were removed and exposed in duplicate to the various developmental conditions.

Table V summarises the experiment, the results being set out so that each horizontal line refers to eggs exposed to a particular set of developmental conditions, the history of their deposition being different in each case, whilst each vertical column refers to eggs with identical histories as regards their

deposition and with varying developmental histories. The results suggest the following conclusions:

(1) The rate of development at a particular temperature is not greater in eggs laid under conditions of high temperature than in those laid under low temperature conditions, *i.e.* a high temperature during oviposition does not result in an accelerated rate of development of the egg.

(2) Under optimum conditions (25° C., 100 R.H.), the rate of development at one temperature is markedly affected by the humidity conditions under which oviposition occurred: it is retarded when these were low, irrespective of what the temperature had been. This effect is not apparent under suboptimal temperature conditions for development, *i.e.* room temperature (R.T.) 100 R.H. Under suboptimal developmental conditions, both of temperature and humidity, there is a differential viability exhibited by eggs laid under different

Table V. *The effect of oviposition-conditions on the subsequent history of the eggs*

Developmental conditions	Time for development in days			
	Oviposition conditions			
25° C. 80 R.H.	Died	Died	Died	Died
25° C. 100 R.H.	27, 29	23, 24	24, 24	28, 28
R.T. 100 R.H.	Killed by mould	36, 37	40, 40	38, 39
R.T. 80 R.H.	Died	39, 44	47, 48	Died
	R.T. 80 R.H.	R.T. 100 R.H.	25° C. 100 R.H.	25° C. 80 R.H.

R.T. = room temperature, which fluctuated from 17 to 23° C., being mostly 20° C.

humidity conditions, irrespective of the temperature at which they had been laid; that is, exposure of the female during oviposition to low humidities adversely affects the viability of the eggs produced.

(*d*) *Velocity of development of eggs at different temperatures*

In the above experiment, the room temperature fluctuated between 17 and 23·5° C. Egg clusters laid at this temperature in saturated air and left to develop began hatching in 36 and 37 days. The general run of temperature over the 37 days may be gauged from the distribution:

17°	17·5°	18°	18·5°	19°	19·5°	20°	20·5°	21°	21·5°	22°	22·5°	23°	23·5° C.
2	1	2	1	2	4	11	1	4	2	1	2	3	1

The mean temperature was 20·25° C. That is, eggs at 20·25° C. hatch in 36 days. We have seen that at 19·2° C. eggs hatched in 43 days.

In Table IV four comparable results are given. The eggs were of the same age, 0–4 days old, and were exposed at the same time. The results are 30, 24, 20 and 18 days for temperatures of 22·5, 25, 27·5 and 30° C. respectively.

If we assume the threshold of development to be 15·5° C., the heat increment in terms of effective day-degrees necessary to effect completion of development at each of these different temperatures is as follows:

Temperature °C.	Days	Effective day-degrees
30	18	261
27·5	20	240
25	24	228
22·5	30	210
20·25	36	170
19·2	43	160

It is clear that the necessary increment of effective day-degrees—the "thermal constant"—is not a constant value, but increases with higher temperatures. That is, the developmental value of a day-degree becomes less as the temperature rises. This is in agreement with the result obtained for the developing larva.

An interesting point in this connection is that at the last two temperatures the necessary heat increment is markedly less, even relatively speaking, than at the other temperatures. These two temperatures represent the mean values of fluctuating temperatures. It is possible that fluctuating temperatures result in an acceleration of developmental velocity, as compared with stable temperatures amounting to the same total in terms of day-degrees. No evidence of such an acceleration, however, was obtained in the case of the development of gorged larvae at fluctuating as opposed to stable temperatures.

IV. Discussion

This paper forms the third of a series of laboratory studies on the relation of the tick, *Ixodes ricinus*, to its physical environment, the two factors chiefly considered being temperature and moisture. In the first two papers (1934, 1935) were recorded the effects and intereffects of these factors on development of the gorged ticks and on general activity and survival of the unfed ticks. The present paper deals with the relation of climate to reproduction, including development of the egg.

Perhaps the most interesting feature of the results is the much greater significance of the humidity than of the temperature factor in the control of reproduction. Apart from the adverse effects of extreme temperatures, near the limits of the range, the latter factor does not appear to exercise any profound effect on the physiological activities associated with reproduction. Thus, it does not appear to influence significantly the maturation of the ovary or its total output, nor does its impress during oviposition materially affect the subsequent history of the egg. The humidity factor, on the other hand, appears to be intimately related to the course and extent of certain of these processes. It would seem to control directly the total egg output of which the ovary is capable. Further, the humidity conditions obtaining at the time of oviposition influence strongly the subsequent fate of the egg, both as regards its viability and its rate of development.

In so far as the extent of the favourable ranges of these factors is concerned, the humidity requirements are similar to those for development, *i.e.* a restricted range of high moisture content. The temperature range, on the

other hand, shows some interesting departures from the limits required for development. The results suggest that the metabolic activities of the period between completion of engorgement and initiation of oviposition can proceed, to a limited extent, at temperatures as low as 2–3° C. Oviposition can proceed at temperatures well below the threshold of development, *i.e.* at 10° C., while the upper temperature limit for this process is distinctly lower than that for development, lying between 27·5 and 30° C. The limits for development of the egg approximate to those for development of the gorged stages. The threshold appears to lie about 15–16° C.; the upper limit of temperature, however, is slightly lower than that determined for the gorged larva.

It is of interest that the temperature range favourable to reproduction should correspond more closely to the range favourable to general activity than to that for development. Other workers have recorded a similar finding for insects. The possible significance of this in relation to the seasonal life cycle of the tick will be discussed in a later paper, in which it is intended to correlate the various laboratory findings with field observations on the distribution and activity of the tick.

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SOME ADULT AND LARVAL CHARACTERISTICS OF A BRITISH "AUTOGENOUS" STRAIN OF *CULEX PIPIENS* L.

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About thirty years ago, Dupree and Mitchell, after investigating (at Baton Rouge, U.S.A.) the habits of *Culex pipiens*, stated not only that this species was "an evil biter", but also that "bred specimens which were never allowed to make a meal of blood, mated and laid fertile eggs" (Mitchell, 1907).

Neumann (1912) published an account of experiments in which several generations of *C. pipiens* were reared upon a diet consisting exclusively of sugar solution. During the ensuing seventeen years, no further researches in this direction appear to have been described.

In 1929, independent communications from Roubaud (1929, 1930), de Boissezon (1929) and Huff (1929) directed attention to the fact that certain "strains" of *C. pipiens* are able (*a*) to breed, under suitable conditions continuously throughout the winter, (*b*) to mate in a confined space[1], and (*c*) to lay fertile eggs without having had a preliminary meal of blood.

Roubaud considered these biological peculiarities to be indicative of a distinct "race" of *C. pipiens*, occurring chiefly in urban areas where large numbers of artificially heated buildings provide suitable winter quarters. Roubaud selected the adjective "autogenous" to distinguish the race in question; the ordinary (blood-egg) race thus becoming the "anautogenous" one. He subsequently (1933) named the two races, respectively, *C. pipiens autogenicus* and *C. pipiens pipiens*.

De Boissezon (1933, 1934), on the other hand, maintains that females of *C. pipiens* (whether town or country bred) exhibit "autogenous" characteristics if the food of the larvae contains a high proportion of iron.

More recently, autogenous strains of *C. pipiens* have been described from Germany, by the late Dr Malcolm MacGregor (1932); and from Greece, Hungary and Malta by Vincent (1933) and Tate and Vincent (1934). Although females of autogenous strains of *C. pipiens* are able to lay eggs without a previous meal of blood, both Roubaud (1930) and Vincent (1933) have independently noted that autogenous females readily bite either men or animals.

In this connection, it is important to note (for reasons which will appear later), that, in a town in France, Legendre (1931, 1932) observed *C. pipiens* breeding freely in flooded cellars, rain-water cisterns and cesspools. These adults had a light-coloured thorax, and the females attacked human beings

[1] This faculty has been termed *stenogamy*, and the absence of it, *eurygamy*.

voraciously. Legendre did not suggest that these adults belonged to an autogenous race, but he referred to the prevalence, in the same district, of a different race of *C. pipiens*, having a dark thorax and breeding in open waters, which did not attack man. It is interesting to compare Legendre's observations with those of Grassi (1923) who noted, some years previously, that, in districts where *C. pipiens* readily attacked human beings, the adults were lighter in colour than those in districts where no such annoyance was experienced.

We are now maintaining, in the laboratory of our Institute, a strain of *C. pipiens* which exhibits all the autogenous characters specified by Roubaud and others. This strain—the first to be recorded in Britain (Marshall and Staley, 1935)—is derived from an egg-raft found, on October 6th last, in an outdoor tank in which a species of *Chara* is being cultivated.

This raft was transferred to a laboratory vessel filled with ditch water, into which fragments of wholemeal bread were introduced from time to time. The eggs hatched after an interval of two days, and the majority of the larvae reached the fourth instar about a fortnight later. The larvae and some of the water were, at the end of October, put into a small breeding jar, which was thereafter kept in close proximity to some heating pipes. Adults commenced to appear on November 12th. None of the females was given a blood meal, but a small egg-raft appeared in the jar in the evening of November 23rd, and a second one on the following morning. Both these rafts (one composed of 38, and the other of 40 eggs) hatched three days later. Up to the time of writing, we have reared four generations of this strain upon a purely vegetarian diet. The adults (like those of the Continental autogenous strains previously mentioned) are decidedly stenogamic, the breeding jars employed providing a mating space of not more than 600 c.c. The Hayling autogenous females also resemble the Continental ones by reason of the readiness with which they attack human beings.

The egg-rafts laid by these autogenous females are much smaller than those laid by anautogenous ones. In the ordinary anautogenous strain of *C. pipiens*, the number of eggs composing the raft usually falls between 150 and 300; whereas, in the Hayling autogenous strain, the number rarely exceeds 110. In 43 rafts of the latter strain, the number of component eggs was found to vary from 37 to 125, with an average of 85.

Hitherto, as will have been noted, the separation of autogenous and anautogenous strains of *C. pipiens* has been effected exclusively by reference to certain biological characteristics exhibited by the adults. Roubaud and Toumanoff (1930) considered the two strains to be morphologically identical; and, so far as we are aware, no evidence to the contrary has yet been submitted.

In order to discover whether any morphological peculiarities distinguish our Hayling autogenous material, it was first of all necessary to obtain some uniformly anautogenous material with which to contrast it. A few larvae of *C. pipiens*, collected in November last, which had developed into adults with obvious anautogenous characteristics, were available for purposes of com-

parison; and through the kindness of Dr Tate a large number of adults and larvae of a known anautogenous strain of this species were obtained from Cambridge.

The results of comparing (i) the Hayling autogenous, (ii) the Hayling anautogenous, and (iii) the Cambridge anautogenous specimens of *C. pipiens* indicate, in our opinion, that not only the adults (of both sexes) but also the larvae of our autogenous strain possess definite diagnostic characteristics. The adults (of both sexes) are separable from those of the anautogenous strains examined by having (*a*) a light-coloured thorax, and (*b*) a larger average number of hairs on the lobes of the ninth tergite. The data on which the latter statement is based are summarised in Table I.

Table I. *Chaetotaxy of lobes of ninth tergite in autogenous and anautogenous strains of* Culex pipiens *L.*

	Autogenous (Hayling)		Anautogenous (Hayling)		Anautogenous (Cambridge)	
	♂	♀	♂	♀	♂	♀
Number of cases examined	100	78	24	24	151	80
Average number of hairs on a lobe	13·3	12·9	8·8	8·0	8·2	8·8

In male adults, an additional diagnostic indication (*c*) is provided by the relative length of the maxillary palp. In the Hayling autogenous strain, the combined length of the first four segments of the palp does not exceed the overall length of the proboscis: whereas, in anautogenous males, the reverse is the case. If the overall length of the proboscis be taken as 100, then the combined length of the first four palp segments varies from 92 to 100 in the Hayling autogenous strain, and from 101 to 109 in both the Hayling and the Cambridge anautogenous strains[1].

It is probable that males of the Hayling autogenous and anautogenous strains are also separable by hypopygial differences: the first division of the mesosome appearing to be broader and more deeply pigmented in the former strain. Further investigations in this direction are now in progress.

Fourth instar larvae of the Hayling autogenous strain differ from those of the anautogenous strains examined in having (*d*) a larger average number of branches in each of the four siphonal tufts, and (*e*) a considerably lower siphonal index.

Data regarding the siphonal chaetotaxy of (i) the Hayling autogenous strain, (ii) the Hayling anautogenous strain, and (iii) the Cambridge anautogenous strain are summarised and compared in Table II. These figures show that, in the specimens examined, tufts with more than three branches were found chiefly, and tufts with more than four branches exclusively, on larvae of the Hayling autogenous strain.

[1] In the Greek autogenous strain the combined length of the first four palp segments of the male varies from 86/100 to 92/100 of the overall length of the proboscis.

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Table II. *Siphonal chaetotaxy of autogenous and anautogenous strains of* Culex pipiens *L. (fourth instar)*

NOTE. The figures in the first column indicate the number of branches in one or other of the four siphonal tufts; and those in the remaining columns the number of occasions on which each particular number of branches was recorded. The Roman numerals denote the four tufts, the one nearest to the base of the siphon coming first.

Number of branches	Hayling autogenous				Hayling anautogenous				Cambridge anautogenous			
	i	ii	iii	iv	i	ii	iii	iv	i	ii	iii	iv
1	—	—	—	—	—	—	2	—	—	—	—	—
2	5	3	10	9	33	32	34	18	60	77	84	71
3	28	29	69	64	10	10	3	21	61	45	25	61
4	45	47	17	24	1	—	—	2	4	3	1	—
5	19	16	3	3	—	—	—	—	—	—	—	—
6	4	5	1	—	—	—	—	—	—	—	—	—
7	—	1	2	—	—	—	—	—	—	—	—	—
8	1	—	—	—	—	—	—	—	—	—	—	—
Total number of cases examined	102	101	102	100	44	42	39	41	125	125	110	132
Average number of branches	4·0	3·9	3·2	3·2	2·3	2·5	2·0	2·6	2·5	2·3	2·2	2·5

On comparing Tables I and II, it will be noted that the average number of hairs recorded in the former, and the average numbers of tuft branches recorded in the latter, amount to about half as much again in the autogenous strain as in the anautogenous ones.

As regards the diagnostic evidence provided by the siphonal index, the examination of 155 larvae of the Hayling autogenous strain showed the average value of the index (as well as its actual value in 58 cases) to be 4·0. In 142 cases the value of the index fell within the limits of 3·7 and 4·3. In 104 larvae of the Cambridge anautogenous strain, the average value of the index (as well as its actual value in 40 cases) was 4·8. In 84 of these cases the index fell within the limits of 4·6 and 5·3. In larvae of the Hayling anautogenous strain, the average value of the index was 5·3: this figure was obtained, however, by examining 20 specimens only[1].

As previously mentioned, the egg-rafts of Hayling autogenous females are composed of much fewer eggs than those of anautogenous ones; the number rarely exceeding 110 in the former case, and usually falling between 150 and 300 in the latter one. For information which enables eggs to be classified individually we are indebted to Dr Tate, who recently pointed out to us that eggs laid by English anautogenous females are more slender than those laid

[1] It seems probable that both the autogenous and anautogenous groups of *C. pipiens* are doomed to progressive subdivision. Roubaud, for instance, has described (in addition to his stenogamic, autogenous *C. pipiens autogenicus* and his eurygamous, anautogenous *C. pipiens pipiens*) a stenogamic, anautogenous *C. pipiens berbericus*, having a somewhat shorter siphon than *C. pipiens pipiens*. The siphonal index of *C. pipiens berbericus* is said to be "about 4·5", while that of the Cambridge anautogenous specimens averages 4·8. It may also be mentioned that although the larvae of the Hayling and of the Greek autogenous strains have the same siphonal index (namely 4·0) they differ from one another in certain respects. For one thing, a large proportion of the larvae of the Greek strain have only three pairs of siphonal tufts.

by descendants of Continental autogenous ones. To ascertain whether eggs of the Hayling autogenous strain could be recognised by this means, measurements of 60 of these eggs were compared with those of a similar number of eggs assumed (on account of being taken from rafts composed of more than 200 eggs) to be of anautogenous origin: the ratio of greatest breadth to overall length being computed in each case. It was found that, in the autogenous eggs, the average value of the ratio (as well as its actual value in 32 cases) was 0·24; whereas, in the anautogenous eggs, the average value of the ratio (as well as its actual value in 43 cases) was 0·22.

In the Hayling autogenous strain, as will be remembered, the females have a light-coloured thorax, and readily attack man. In the latter respect they resemble the autogenous females independently investigated by Mitchell (1907), Roubaud (1930) and Vincent (1933): while in both respects they resemble the "cellar-breeding" strain described by Legendre (1931, 1932). In this connection it may be of interest to refer to the fact that, in Britain, where *C. pipiens* is generally regarded as being a "man-ignoring" species, well-authenticated instances of its causing widespread annoyance in urban areas are occasionally recorded. Shute, for example (1933), refers to an invasion, by bloodthirsty females of *C. pipiens*, of certain private hotels in the Charing Cross district, as well as to the occurrence of similar incidents in the residential parts of Plumstead, Hull, Harwich and Epsom. In yet another case (which came under the personal notice of the authors some years ago) a large hotel in Westminster became seriously infested with man-biting females of the above species. A few female adults collected on that occasion were preserved, and an examination of the ninth tergites shows the average number of hairs per lobe to be 12·0; a fact which appears to indicate that the adults are of autogenous origin[1].

We suggest that, in all the above-mentioned cases of indoor annoyance, the offending females of *C. pipiens* very probably belonged to the (or a) British autogenous strain—a strain which may prove to be identical with the cellar-breeding one described by Legendre.

SUMMARY

1. A strain of *Culex pipiens* which is autogenous and stenogamic has been found at Hayling Island, England, and compared with British anautogenous strains.

2. Definite characteristics in adults of both sexes and in larvae have been found which separate the autogenous from the anautogenous British strains of *C. pipiens*.

3. The autogenous strain differs from the anautogenous strains in (*a*) the

[1] A similar inference is perhaps permissible in respect of two small batches of females captured while attacking the occupants of houses in Ventimiglia (Italy) and in Senlis (France); the average number of hairs per lobe being 11·8 in the former batch and 13·0 in the latter one. All these Italian and French adults (as well as the adults from the hotel in London) have a light-coloured thorax.

lighter colour of the thorax of the adults; (*b*) the greater average number of hairs on the ninth tergite of the adults; (*c*) the relative shortness of the male palps in comparison with the proboscis; (*d*) the larger average number of branches in each of the four siphonal tufts of the fourth instar larvae; (*e*) the lower siphonal index of the fourth instar larvae; and (*f*) the greater width of the egg relatively to its length.

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OBSERVATIONS UPON *CHILOMASTIX* FROM *BUFO VULGARIS*, WITH NOTES ON *CHILOMASTIX AULASTOMI*[1]

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(With Plate XVII, containing Figs. 1–32)

CONTENTS

		PAGE
I.	Introduction	507
II.	Material and methods	508
III.	Description of *Chilomastix* from *Bufo vulgaris*	509
IV.	Description of division in *Chilomastix* from *Bufo vulgaris*	511
V.	The cysts of *Chilomastix* from *Bufo vulgaris* and of *Chilomastix aulastomi*	514
VI.	Discussion	516
VII.	Summary	516
	References	517
	Explanation of Plate	518

I. Introduction

With the exception of *Chilomastix gallinarum* and *C. aulastomi* little is known of the method of division of the members of the genus *Chilomastix*. Kofoid and Swezy (1920), however, have described mitosis in the cysts of *C. mesnili* though they did not observe division in the flagellate. The present study is an attempt to work out the method of division and the structure of the cysts of *Chilomastix* from the intestine of *Bufo vulgaris*, and to compare this flagellate with other species of *Chilomastix*, particularly with *C. aulastomi*.

Alexeieff (1909) gave the name of *Macrostoma*[2] (=*Chilomastix*) *caulleryi* to a flagellate which he found in frog tadpoles and in Axolotl. Later (1910) he reports having seen a *Tetramitus* (=*Chilomastix*) resembling *T. caulleryi* (=*C. caulleryi*) in preparations made by Chatton from the gut of *Haemopis sanguisuga*. In a discussion following a paper by Nattan-Larrier (1912) Chatton himself said that he saw an intense infection of *Tetramitus* (=*Chilomastix*) in *Haemopis sanguisuga* at Belfort in 1910. Alexeieff (1914, 1914 *a*) believed that the intestinal faunae of *H. sanguisuga* and Amphibia were

[1] A study begun during my tenure of a Beit Memorial Fellowship and completed since I became a Yarrow Fellow.

[2] The variations of nomenclature through which the genus *Chilomastix* has passed have been dealt with in detail by Chalmers and Pekkola (1918), Dobell and O'Connor (1921) and Wenyon (1926).

identical. But Bělař (1921) regarded the *Chilomastix* from the leech as distinct from *C. caulleryi* and called it *C. aulastomi*. He described division in this species.

Dobell (Dobell and O'Connor, 1921) has studied the morphology of *C. caulleryi*, Wenyon (1926) remarked that he had seen a *Chilomastix*, probably *C. caulleryi*, in the common English toad, and Grassé (1926) described the morphology of *C. caulleryi* from *Rana temporaria*, *Bufo vulgaris*, *Pelodytes punctatus*, *Molge marmorata* and *Pleurodeles Waldtlii*, but none of these writers described division.

II. Material and methods

During my study (Bishop, 1931) of *Trichomonas* from *Rana temporaria* and *Bufo vulgaris*, *Chilomastix* and its cysts were found in the large intestines of some of the toads but never in the frogs. Attempts were made to cultivate the flagellate and growths were obtained in tubes containing inspissated horse-serum slopes covered with inactivated horse-serum diluted 1 : 10 in 0·5 NaCl solution, and in the liquid medium alone. Although, owing to the smallness of the cytopharyngeal opening, *Chilomastix* is unable to ingest solid rice-starch a little was added (sterile) to each tube of medium before inoculation, as *Blastocystis* was invariably present in the faeces of the toads and rice-starch has been shown (Dobell and Laidlaw, 1926) to be very helpful in eradicating this organism. Many attempts were made to obtain "pure" cultures of *Chilomastix* (cultures containing only this one species of protozoan, together with a mixed flora of bacteria), but it was impossible to separate them from *Trichomonas batrachorum* which was present in every toad examined. Every method devised for killing the trichomonads destroyed the *Chilomastix* also. Even attempts to obtain "pure" cultures from cysts by treating them with *N*/20 HCl were frustrated owing to a few trichomonads being able to withstand as big a dose of acid as the cysts. (The resistance of other species of *Trichomonas* to *N*/20 HCl has been described previously (Bishop, 1930).) The trichomonads multiplied more rapidly than *Chilomastix*, so that though the *Chilomastix* persisted for 4–6 weeks in the initial cultures, and were at first very numerous, each successive subculture contained a greater proportion of *Trichomonas* to *Chilomastix*, and the final result was, in every case, a "pure" line of *Trichomonas batrachorum*.

Attempts to cultivate *Chilomastix aulastomi* from the hind-gut of the horse-leech (*Haemopis sanguisuga*)[1] have failed from a similar cause. *Chilomastix aulastomi* was swamped by *Trichomonas sanguisugae*, of which a "pure" strain was obtained (Bishop, 1932).

Chilomastix in the mixed cultures with *Trichomonas* appeared healthy and normal in structure. The flagellates divided actively in the initial and first subcultures and many cysts were found as the cultures grew old. On this material the following study is based.

[1] For the identification of the leeches the paper by Harding (1910) has been used.

Schaudinn's solution, with 4–5 per cent. glacial acetic acid, and Bouin's fluid were used as fixatives, and the preparations were stained with Heidenhain's haematoxylin.

III. DESCRIPTION OF *CHILOMASTIX* FROM *BUFO VULGARIS*

During the last five years I have examined many toads, chiefly from Lincolnshire, Cambridgeshire, and the Exeter district. About 20 per cent. were found to be infected with *Chilomastix*, and in each case the flagellates were numerous and cysts were also present. Many frogs and frog tadpoles have also been examined but *Chilomastix* has never been found in their intestinal contents. Grassé (1926), however, found *C. caulleryi* in *Rana temporaria*.

Chilomastix from the toad is a large flagellate, measuring, in culture, 18–45 × 8–12 μ, the majority of individuals being 25–35 μ long. But in the host they are, on the whole, smaller, their length being 15–30 μ. Alexeieff gives 25 μ as the length of *Macrostoma* (=*Chilomastix*) *caulleryi*, and Grassé 15–30 μ. The shape of the body varies. In the majority of specimens from cultures it is carrot-shaped, being broadest near the anterior end and tapering gradually (Figs. 1, 2 and 3) to form a tail which varies in length. Individuals (Fig. 4) studied directly in the intestinal content of the host were much broader in proportion to their length than cultural forms.

In the living flagellate a spiral groove runs, from right to left, from the anterior end of the body towards the posterior. The part of the body below this groove appears to be twisted. In fixed preparations this groove and the torsion are seldom visible. There is a large cytostome which lies in the anterior third of the body and follows a slightly spiral course in the same direction as the groove. Dobell and O'Connor (1921), in their description of *Chilomastix mesnili*, have termed the side bearing the cytostome ventral, and their terminology is followed here. The body is covered with a delicate pellicle and does not undergo change of shape. Amoeboid forms, such as those described in *C. mesnili* by Brug (1922) from a liquid stool, have never been seen in *Chilomastix* from the toad. A strain of *C. mesnili* was isolated from human faeces by Mr Dobell and myself in December 1927 and cultivated, principally by me, until August 1929, on inspissated whole-egg slopes covered with inactivated horse-serum diluted 1 : 8 in Ringer's solution. During this period amoeboid forms were not seen.

In stained preparations the vesicular nucleus of *Chilomastix* from the toad can be seen lying at the extreme anterior end of the body (Figs. 1–4). There is a well-defined nuclear network on which chromatin granules, of varying size, are scattered irregularly. The bulk of the chromatin is massed in one or two karysomes which lie at the periphery. To the right of the nucleus lie four basal granules from which the flagella arise. In many individuals they form a compact mass and only the most careful differentiation reveals their quadruple nature, but in others they are more widely separated. From three of them

arise the three anterior flagella and from the fourth arises a shorter, very delicate flagellum which lies in the cavity of the cytostome. The continual flickering of this cytostomial flagellum directs a current of liquid, carrying bacteria, to the neighbourhood of the cytopharyngeal opening. Kofoid and Swezy (1920) believe this cytostomial flagellum to be attached to the body by an undulating membrane, but no evidence of this has been found in the many living individuals which I have examined. From its action it appears to be a flagellum lying freely in the cytostomial cavity and comparable with the cytostomial flagellum of *Embadomonas* (Bishop, 1931 *a*, 1934), except that its tip does not project beyond the edge of the cytopharynx as in the latter species. In a *Chilomastix* (Fig. 23) rounded up prior to encysting the cytostomial flagellum projected freely from the cytostome.

Descriptions of the basal granules of *Chilomastix* vary. Wenyon (1910) says that in *Macrostoma* (=*Chilomastix*) *mesnili* the three flagella arise from a deeply staining chromatin granule. Chalmers and Pekkola (1918) describe three basal granules in the same species. Kofoid and Swezy's (1920) description of these granules agrees in number with Chalmers and Pekkola's, but they also describe a centrosome on the surface of the nucleus and rhizoplasts linking up the structures. Boeck (1921) confirms Kofoid and Swezy's findings in his study of *Chilomastix mesnili* in culture. According to Dobell and O'Connor (1921) there are six basal granules in *C. mesnili*, four giving rise to the flagella and the remaining two to the two peristomial fibres. In *C. aulastomi*, Bělař (1921) describes four basal granules and states that the fibrils of the peristome are not connected with them. In *C. caulleryi*, Grassé (1926) found either three or four basal granules. Though, like Kofoid and Swezy, he described connecting rhizoplasts, he did not find a centrosome. In my own experience the basal granules are so small and so close together that it would be impossible to show a system of connecting fibres. Boeck and Tanabe (1926) found four blepharoplasts in *C. gallinarum*, two anterior ones giving rise to the three anterior flagella, and two posterior ones giving rise to the peristomial fibrils and the peristomial flagellum.

The cystome is an elongated depression with well-defined lips. The opening of the cytopharynx lies at its posterior end and is small and can seldom be seen. *Chilomastix*, like *Embadomonas* (Bishop, 1931 *a*, 1934), is unable to digest starch grains. It feeds upon bacteria, and the cytoplasm usually contains many in process of digestion. The lip on the right side of the cytostome is slightly irregular. Its edge stains slightly (Fig. 2), but in individuals from which the stain has been greatly extracted it is not discernible. The right lip is supported by a strong, siderophilic fibril which lies below the surface. This fibril begins at the anterior end of the cytostome (Fig. 2) or a little to its left (Fig. 1), runs a slightly sinuous course to the posterior end of the cytostome round which it curves, and curving again spirally upwards it encircles the cytopharynx almost completely, and ends deeply in the cytoplasm. The left lip is supported by a more delicate fibril which lies almost flush with the

surface and gradually tapers away in the region of the cytopharynx. In some positions of the flagellate the posterior end of the left fibril (Fig. 3) appears superimposed upon the upward curve of the posterior end of the right cytostomial fibre, giving the false impression that the two fibrils are continuous. In many individuals (Figs. 1, 2 and 4) there is a more deeply staining area below the right fibril which gives the impression of a second and deeper fibril. This is particularly noticeable in some stages of division. Its presence is not sufficiently constant for it to be regarded as a definite part of the adult cytostome. In the fully grown flagellate the peristomial fibrils are not connected with the basal granules of the flagella.

In *C. mesnili* Kofoid and Swezy (1920) describe, in addition to the right and left peristomial fibres, a fine fibre encircling the rim of the cytostome. They call the right peristomial fibril the parabasal and the left the parastyle. Chalmers and Pekkola (1918) and Kofoid and Swezy (1920) both homologise this "parabasal" with the organ they call the "parabasal" in *Trichomonas*. This organ in *Trichomonas* is not a true parabasal but the basal fibre supporting the undulating membrane and its flagellum (Bishop, 1931); a true parabasal exists as well (Grassé, 1926). Grassé has also shown the presence of a true parabasal in *Chilomastix caulleryi*. Chalmers and Pekkola's and Kofoid and Swezy's homology rests upon the assertion (which my observations have failed to confirm) that the peristomial flagellum is attached to the base of the peristome by a membrane. Bělař's description of the cytostome of *C. caulleryi* agrees in all essentials with my description of *Chilomastix* from the toad.

IV. DESCRIPTION OF DIVISION IN *CHILOMASTIX* FROM *BUFO VULGARIS*

The most frequent stage of division found in the cultures of *Chilomastix* from *Bufo vulgaris* shows (Figs. 5 and 6) two basal granules at either side of the nucleus with two of the flagella attached to one pair of granules and one to the other. The body has become rounded but the nucleus remains in its original position. The cytostome appears to have shrunk, and though the right and left fibrils are distinct the cytostomial flagellum is invisible. The right and left cytostomial fibrils of a new, and not wholly differentiated, cytostome appear to be attached to, or to lie close to, the basal granules on the side of the nucleus opposite to the original cytostome. In the living flagellate the cytostomes appear as pits at the bases of the flagella. As division progresses they gradually elongate towards the centre of the body. Although the peristomial fibres in the undividing flagellate are not connected with the basal granules, they appear, in both the original and the new cytostome, to lie in close contact with them during division.

The chromatin becomes massed in the centre of the nucleus in one or two large granules. A faintly staining spindle (Fig. 7) forms between the two pairs of basal granules and faint spindle fibres are sometimes seen. The nuclear mem-

brane persists. The chromatin becomes arranged as one or two large blocks at the equator. The spindle elongates and extends across the body (Figs. 8 and 9). The chromatin begins to move towards the poles as either one (Fig. 10) or two (Fig. 11) large blocks, but their division has not been seen. The number of "chromosomes", therefore, if these large blocks of chromatin can be called such, appears to be variable. At the anaphase (Figs. 11 and 12) a third basal granule appears at either pole, but their origin is obscure. Cytostomial flagella now become visible in both cytostomes, and a second anterior flagellum grows out from the pole which bore only one. The structure of the cytostomes becomes more definite, and a granular line is frequently visible below the right peristomial fibre (Figs. 11 and 13). The nuclear membrane, which is faintly siderophilic, becomes constricted in the middle, and then forms (Figs. 12 and 13) a deeply staining thread between the daughter nuclei. If the earlier stages of division were not known this thread might be mistaken for a centrodesmose. It finally disappears (Figs. 14 and 15). The daughter nuclei each contain one large karyosome. The polar parts of the spindle persist for some time after a complete membrane has been formed round each nucleus. When their last traces disappear, the nuclear membranes of the daughter nuclei begin to stain intensely (Fig. 15), as a thin layer of chromatin is deposited on their inner surfaces. A few extrakaryosomic granules appear in the nucleus. The peristomial fibres move further away from the basal granules. The body becomes constricted in the middle and, finally, the two daughter flagellates separate (Fig. 16). At the time when division of the body occurs there are usually only two anterior flagella in addition to the cytostomial flagellum, and three basal granules at each pole, the full number appearing later (Fig. 17) during the growth of the daughter flagellates. During division the body is plastic and capable of some change of form.

The majority of individuals in cultures of *Chilomastix* divide in this way but a few stages of division have been found which differ from this type and appear to form a distinct series (Figs. 18–22). Several individuals have been seen in which, though the normal shape of the body persists, the nucleus has migrated into the centre. Such individuals (Fig. 18) contain a single large karyosome centrally placed, and a pair of basal granules which lie opposite to one another on the outer surface of the nuclear membrane. Three flagella remain at the anterior end of the body but their basal granules have disappeared. A fibre is seen in their neighbourhood which may be the remains of the cytostome but no trace of the cytostomial flagellum is visible. In a later stage of this series (Fig. 19) the body is more rounded, and the basal granules can be seen at either pole of what appears to be the beginning of an extranuclear spindle. Later (Fig. 20) an intensely siderophilic spindle-shaped structure is formed between the two pairs of basal granules, but spindle fibres are never seen. New cytostomial fibrils are formed from the pairs of basal granules, while the old flagella and traces of the original cytostome persist. By the anaphase (Fig. 21) the rudiments of the new cytostomes are distinct,

but no new flagella have yet been formed. Even when the daughter nuclei have formed (Fig. 22) the new flagella are still absent.

Cultures from five toads have been examined and in all of them a few individuals have been found dividing by this second method in addition to many dividing by the first. Preparations from the intestinal contents of many toads have been examined, but no stages of division have been found. Grassé (1926) likewise reports that he never saw division in *Chilomastix caulleryi* in the intestinal contents of the Amphibia which he examined.

These two methods of division appear to me to differ in some essential points. In the first type the original cytostome persists throughout the division process and a second cytostome develops from two of the basal granules. The original three anterior flagella are retained attached to the basal granules at the poles of the spindle, and two pass to one daughter flagellate and one to the other. In the second type of division the original cytostome disappears early in the process and two new ones are formed later. The nucleus and the basal granules migrate to the centre of the body, but the flagella remain in their original position, unconnected with the spindle. The appearance of the spindles in the two types of division differs markedly. But in neither type of division is a centrodesmose formed as the four basal granules separate into two pairs to form the poles of the spindle. In this *Chilomastix* differs from *Embadomonas* (Bishop, 1931 *a*, 1934) and *Trichomonas* (Bishop, 1931, 1932) and resembles *Hexamita gigas* (Bishop, 1933).

There are three possible explanations of the existence of these two types of division in cultures of *Chilomastix* from the toad. It may be considered that one type of division is due to a series of abnormalities, or that two types of division are possible in one species, or, thirdly, that two species of *Chilomastix* may be present. That the variations always fall into two definite series seems to argue against the first explanation. Examination of a large number of undividing flagellates of *Chilomastix*, both in cultures and in intestinal contents, failed to reveal differences of specific value, though great variation in size and shape exists. In the cysts no greater difference in shape and size is found (Figs. 25–30) than is normal in a pure species. On comparing the methods of division in *Chilomastix* from the toad with Bělař's account of division in *C. aulastomi*, it is interesting to find a striking resemblance between *C. aulastomi* and the second method of division of *Chilomastix* from the toad. I have been able to confirm Bělař's account of the division of *C. aulastomi* in my cultures (p. 508) of that species mixed with *Trichomonas sanguisugae*. That the second type of division in *Chilomastix* from *Bufo vulgaris* is similar to the only type described from *Chilomastix aulastomi* seems to support the theory that two species of *Chilomastix* are present in the toad.

Though he does not describe division in *C. caulleryi*, Alexeieff (1912, Fig. 2) figures a stage of division in this flagellate (from Axolotl) which is essentially similar to *C. aulastomi* at the same stage of division and also to the second type of division in *Chilomastix* from the toad.

Apart from *C. aulastomi* the only other species of *Chilomastix* in which division has been described in any detail, in the flagellate stage, is *C. gallinarum* (Boeck and Tanabe, 1926). In this species the original cytostome persists during division and a second cytostome is formed at the time of the anaphase. The authors believe that the blepharoplasts divide at the time of the prophase and that one group, with either one or two of the anterior flagella, migrates and at the later prophase is found at the distal end of the spindle. As in *Chilomastix* from the toad and in *C. aulastomi* no centrodesmose is formed. It is difficult, from their figures, to arrive at any conception of the exact behaviour of the flagella as at one early stage (Fig. 6) the total number is greater than at most of the subsequent stages (Figs. 7, 8 and 9). Division in *C. gallinarum* resembles the first type in *Chilomastix* from the toad in that the cytostome and flagella are not absorbed.

V. The cysts of *Chilomastix* from *Bufo vulgaris* and of *Chilomastix aulastomi*

The cysts of *Chilomastix* from the toad, like those of *C. mesnili*, are oval to pear-shaped. They measure 8–12 × 6–8 μ. According to Alexeieff (1912) the cysts of *C. caulleryi* measure 7 × 5 μ. In the living cyst the wall has a sharp double contour, but, with the exception of a few refringent granules, no other internal structure can be made out. Cysts stained with iodine show a single nucleus and the cytostomial fibrils. Glycogen reserves have not been seen. In cysts stained with Heidenhain's haematoxylin the internal structure appears complex. In many individuals there is a slight space between the protoplasm and the cyst wall (Figs. 26 and 27). The nucleus is frequently well-defined (Figs. 25, 26 and 27) and lies, in the majority of cysts, near the centre, though sometimes (Figs. 25 and 29) it is at the anterior end. The nuclear membrane is usually visible, and in most cysts the chromatin is concentrated in a large karysome of irregular shape. Occasionally the karyosome is small (Fig. 25) and there are many extrakaryosomic granules. In some cysts it is double (Fig. 27) and in a few dumb-bell-shaped (Fig. 30) as though about to divide. But though the karyosome may occasionally divide or fragment, no other sign of division or duplication of organs has been found in the many cysts studied.

Many deeply staining granules are frequently present, and this makes the interpretation of the internal structure difficult. In many cysts, however, the four basal granules can be seen and in some cysts they are very distinct. Frequently they lie towards the more pointed (anterior) end, but in a few (Fig. 25) they were found towards the posterior end, and in such cysts the position of the peristomial fibrils is also reversed. Kofoid and Swezy (1920) describe a similar reversal of position in these organs in a few cysts of *C. mesnili*. In most cysts the right peristomial fibril is very distinct, but sometimes it is partly obscured by the twisted strands of the anterior flagella. In only a few cysts has it been possible to make out the left fibril. In addition to the peri-

stomial fibrils the anterior flagella are present, curled round inside the wall. Their course may be extremely difficult to follow, as they are frequently much twisted together.

Flagellates (Figs. 23 and 24) have been found, in cultures rich in cysts, in which the body is rounded up and the nucleus contains a large karyosome. Such individuals are probably precystic stages.

The cysts of *C. aulastomi* (Figs. 31 and 32) measured 6–8 × 5–7 μ and are therefore smaller than those of *Chilomastix* from *Bufo vulgaris*. They were also more globular than cysts of *Chilomastix* from the latter host. They had a thin wall. The nucleus was similar in position to that in cysts of *Chilomastix* from the toad. The four basal granules were sometimes visible. The right peristomial fibril was well defined, as also were the anterior flagella. Siderophilic granules were few or absent.

Several workers have described cysts of *Chilomastix*, and some believe that division occurs in this state. In the cysts of *C. mesnili* from *Box salpa*, Alexeieff (1912) portrays a nucleus, cytostome, and other fibrillar structures which he does not name. He found similar structures in *Chilomastix caulleryi*. In *Tetramitus* (= *Chilomastix*) *mesnili* from man, Wenyon and O'Connor (1917) describe a single nucleus and one or more granules near the anterior end from which the cytostomial fibre and the cytostomial flagellum (when visible) take origin. Chalmers and Pekkola's (1918) description is similar, but in one cyst the karyosome was dumb-bell-shaped and this they interpret as an early stage in division. They state that they saw two nuclei in another cyst, but their photomicrographs are so indistinct that they give little support to this statement. In the same species Kofoid and Swezy (1920) found the basal granules and a system of rhizoplasts, the buccal apparatus and the nucleus, but they state that the flagella were not present. They claim to have found mitosis in the cysts and figure the stages. Their description of the division of the nucleus differs from all descriptions of nuclear division in the flagellate stage of *Chilomastix* in that they state that a centrodesmose is formed between the daughter centrosomes. Dobell and O'Connor (1921) never saw mitosis in the large number of cysts upon which their study was based. They criticise the possibility of demonstrating so much detail in so small a cyst. They themselves found a single nucleus, the peristomial fibrils, and six blepharoplasts in the cysts. Hegner (1923) defends Kofoid and Swezy's description of mitosis in the cysts and says that he found binucleate cysts and a number of stages in division of the nucleus. Leiva (1921) states that he has found a telophase in one cyst of *C. intestinalis* from the guinea-pig. In cysts of *C. gallinarum* Boeck and Tanabe (1926) describe a nucleus, a centrosome, blepharoplasts, and the cytostome and its fibres, but no flagella. They state that though they have examined a considerable number of cysts they "have not encountered a single one showing any indication of division". Bělař's (1921) figure of *C. aulastomi* differs from those found in my cultures. It is pear-shaped, as are the cysts of *C. mesnili* and many of the cysts of the *Chilomastix* from the toad. A single

nucleus and the right cytostomial fibril are shown, but there is no indication of the flagella.

I have examined many cysts of the *Chilomastix* from the toad and also of *C. aulastomi* without finding any containing stages of division or two nuclei. From the evidence available, therefore, division in the cysts of *C. mesnili* or other species of *Chilomastix* must be very rare.

VI. Discussion

This study of *Chilomastix* from *Bufo vulgaris* raises the question whether one or two species are present in this host. No specific differences have been found in the undividing flagellates or their cysts, but two methods of division were found in cultures of the flagellates. In the type of division seen most frequently (first method) the original cytostome and the flagella persisted throughout the whole process. In this it resembled *Chilomastix gallinarum* as described by Boeck and Tanabe (1926). In the second type of division, which was rare, the original cytostome was absorbed and entirely new ones were formed, this type being similar in all essentials to that of *C. aulastomi* from the leech as described by Bělař and confirmed by me. Alexeieff (1914, 1914 *a*) believed that the faunae of *Haemopis sanguisuga* and Amphibia were identical. Bělař, however, regarded the *Chilomastix* from the leech (*C. aulastomi*) as distinct from *C. caulleryi*. From the similarity of their mode of division it is possible that the *Chilomastix* from the leech and that *Chilomastix* from the toad which divides by the second method are of the same species. If further investigation should prove this identity it might be presumed that *Chilomastix* from the toad which divides by the second method is *C. aulastomi*, and that that *Chilomastix* from the toad which divides by the first method is *C. caulleryi*. But the matter is complicated by Alexeieff (1912) having figured a stage of division of *C. caulleryi*, and this stage is similar to one found in the second type of division of *Chilomastix* from the toad and therefore to *C. aulastomi*. If therefore the *Chilomastix* from *Haemopis sanguisuga* and the *Chilomastix* from the toad dividing by the second method are one species, then *C. aulastomi* will be a synonym of *C. caulleryi*, and that *Chilomastix* from the toad which divides by the first method will be a new species.

VII. Summary

1. *Chilomastix* from *Bufo vulgaris* has been grown in culture media but a "pure" strain was not obtained. Division and encystment occurred.

2. Two types of division were found. In one type the original cytostome and flagella persisted and a new set was formed from one pair of basal granules. In the other type the original cytostome disappeared and two new ones were formed from the two pairs of basal granules. This second type of division is similar to that of *Chilomastix aulastomi* from *Haemopis sanguisuga*.

3. It is therefore probable that two species of *Chilomastix* are present in *Bufo vulgaris*.

4. Cysts of *Chilomastix* from *Bufo vulgaris* and of *Chilomastix aulastomi* were studied. Nuclear division was never seen in them.

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EXPLANATION OF PLATE XVII

The figures were drawn with an Abbe camera lucida on a Leitz binocular stand, with a 1/12 in. fluorite oil-immersion lens. Figs. 1–23 were drawn with a ×12 periplanatic ocular at an approximate magnification of 2000 diameters. Figs. 24–31 were drawn with a ×20 periplanatic ocular at an approximate magnification of 3300 diameters. The figures have not been reduced.

The flagellates were fixed with Schaudinn's or Bouin's fixative and stained with Heidenhain's haematoxylin.

Chilomastix from *Bufo vulgaris*, grown in culture

Figs. 1–3. Individuals showing nucleus, cytostome, cytostomial flagellum, three anterior flagella, four basal granules, and ingested bacteria.

Fig. 4. *Chilomastix* from the intestinal content of *Bufo vulgaris*.

Figs. 5, 6. Prophase. Two cytostomes are already present.

Figs. 7–9. Stages in spindle formation. Two basal granules are present at either pole. Two flagella are at one pole and one at the other.

Fig. 10. Anaphase with one "chromosome" going to each pole.

Fig. 11. Same with two "chromosomes". Three basal granules at the poles.

Fig. 12. Nuclear membrane constricting. Three basal granules and two flagella at either pole.

Fig. 13. Reorganisation of daughter nuclei.

Fig. 14. Same.

Fig. 15. Polar area of spindle has now disappeared.

Fig. 16. Cytoplasmic division.

Fig. 17. Division completed. Development of third flagellum.

Figs. 18–22. Second type of division of *Chilomastix* from the toad, grown in culture. Disappearance of the original cytostome and growth of new ones from the two pairs of basal granules.

Figs. 23–24. Precystic forms.

Figs. 25–30. Cysts.

Fig. 25. Reversal of position of basal granules.

Fig. 30. Dumb-bell-shaped karyosome.

Figs. 31–32. Cysts of *Chilomastix aulastomi* in culture.

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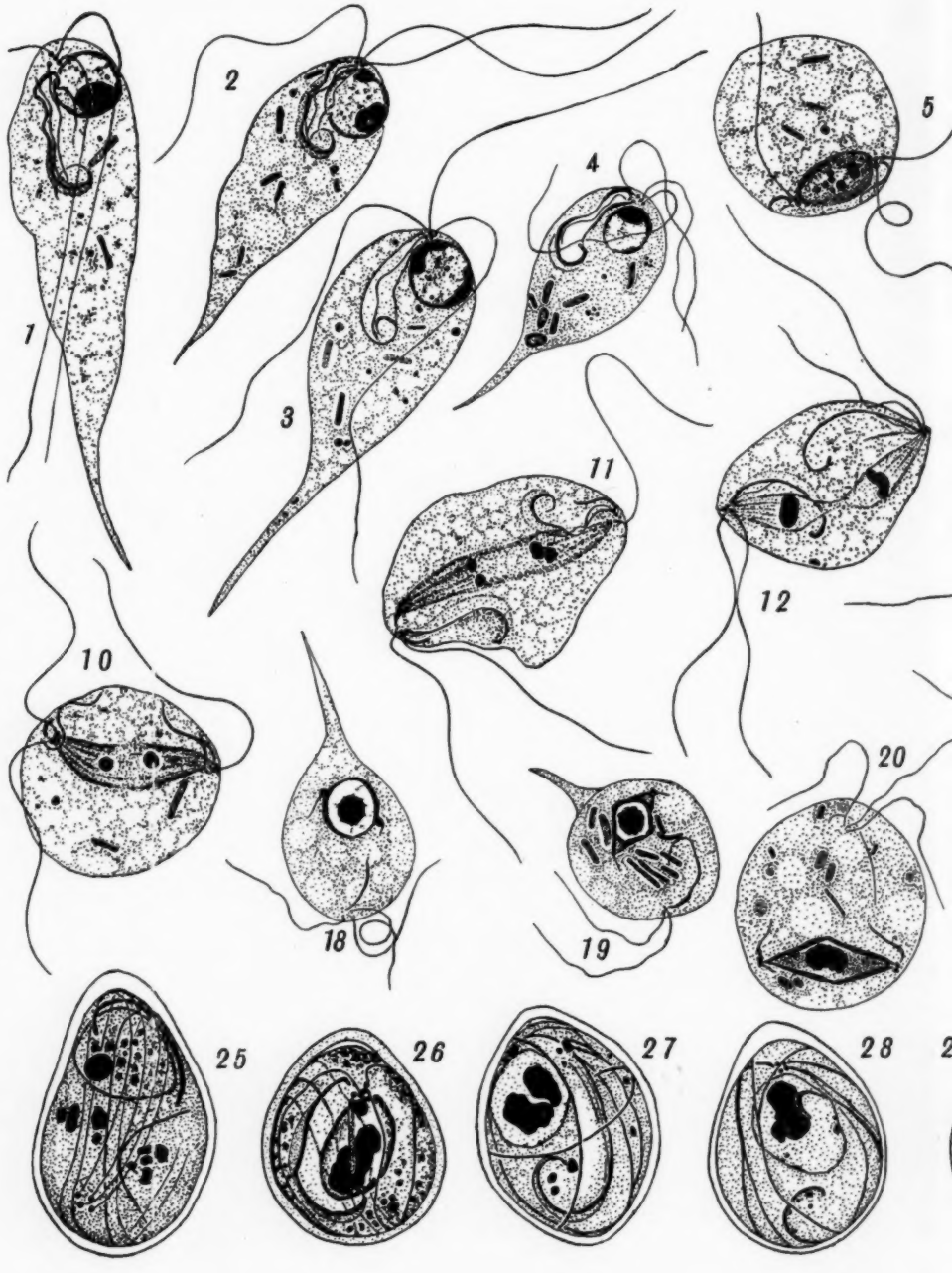
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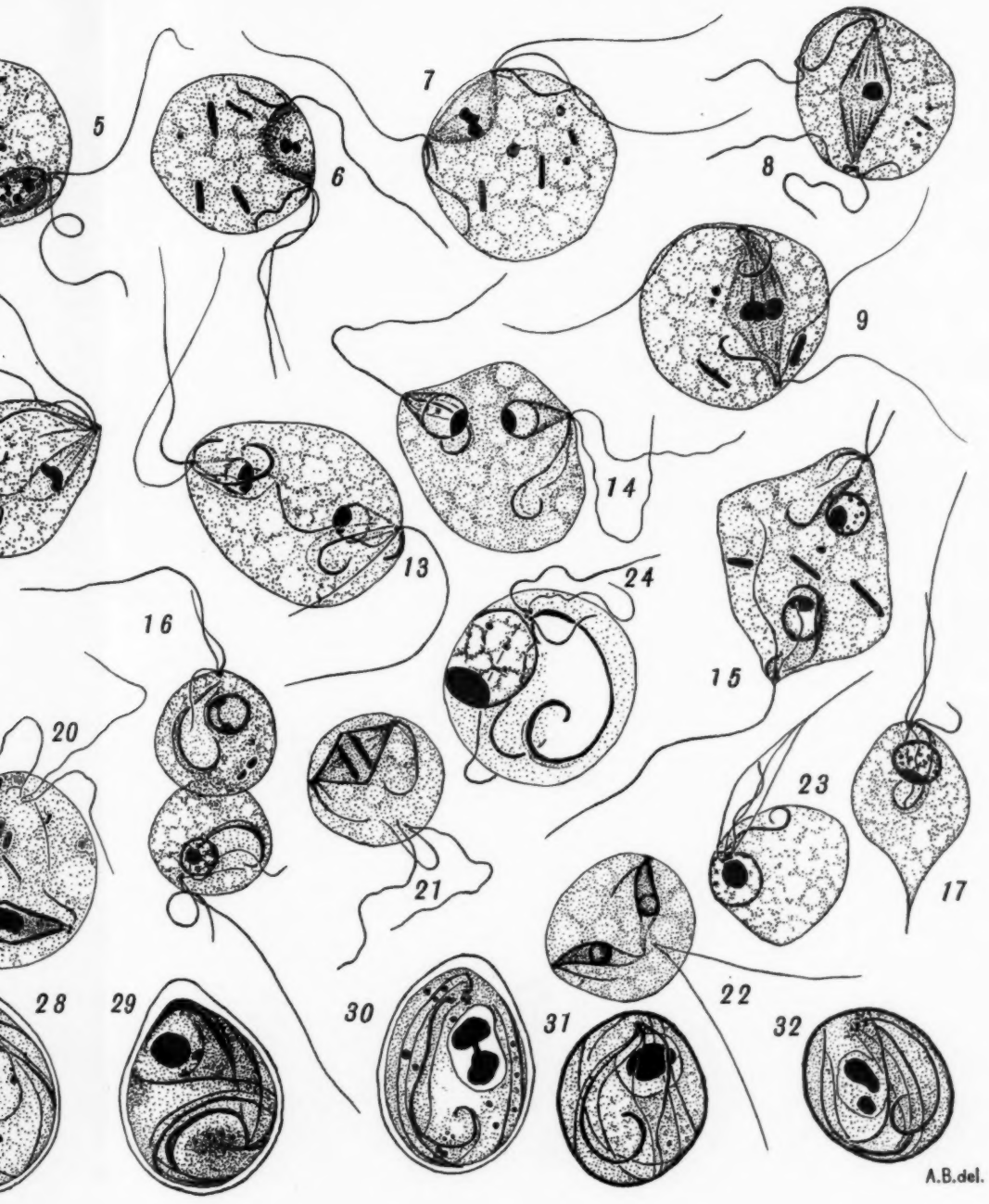
PLATE XVII



A.B.del.

DESCRIPTION OF A TICK, *ORNITHODOROS COPROPHILUS* N.SP., FROM BAT GUANO

By ALLEN McINTOSH

From the Zoological Division, Bureau of Animal Industry, U.S. Department of Agriculture, Washington, D.C.

(With Plate XVIII and 2 Text-figures)

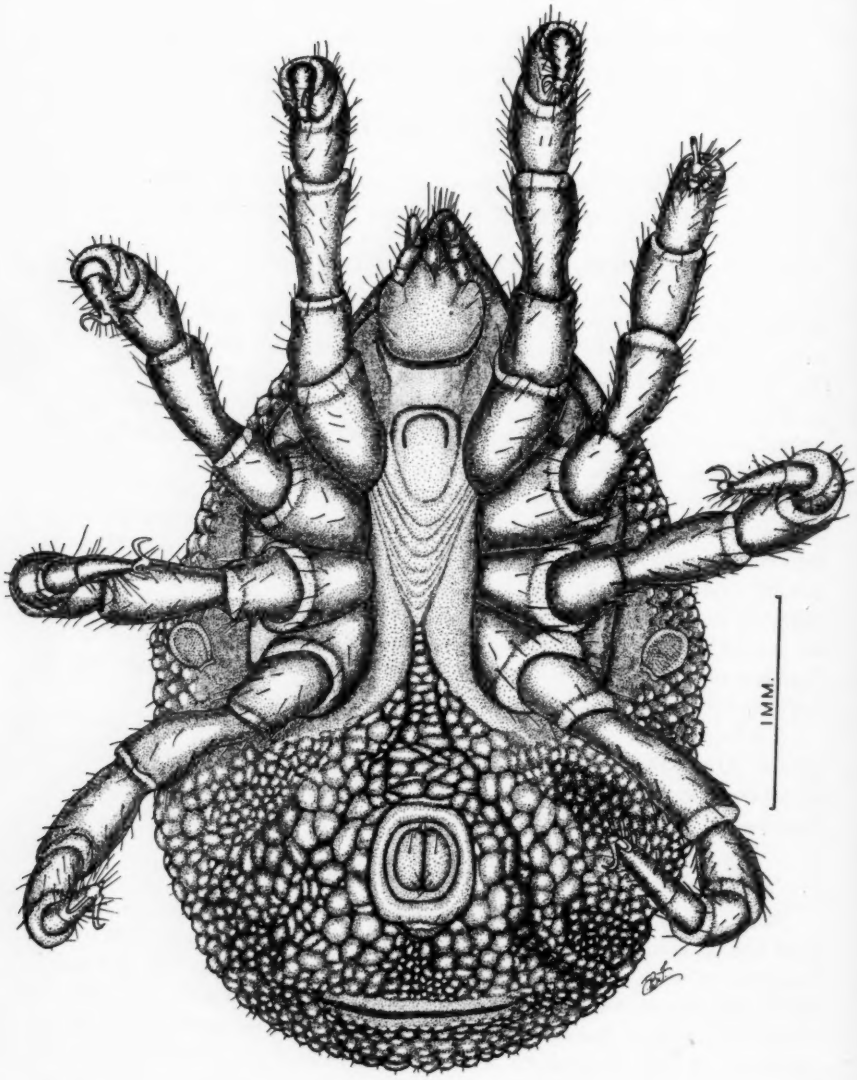
In October, 1934, several specimens of a tick belonging to the genus *Ornithodoros* were forwarded to the Bureau of Animal Industry for identification by Drs William Thompson and N. E. Dutro. These ticks were first observed by Mr F. E. Swan of the Bureau of Entomology and Plant Quarantine in a carload of bat guano originating at Linares, Mexico, and held in quarantine at Matamoros, Mexico.

To facilitate identification of the above specimens, the writer was permitted, through the courtesy of Dr H. E. Ewing, to examine several lots of *Ornithodoros* which were in the collections of the U.S. National Museum. Among this material were several specimens labelled *O. talaje* which were collected May 29th, 1931, by Prof. L. P. Wehrle, University of Arizona, from bat guano at Tucson, Arizona. On examination these were found to be not *Ornithodoros talaje* (Guérin-Méneville 1849) but specimens of the same species as those taken from the bat guano from Linares, Mexico.

A comparison of the species from Linares, with descriptions of the other species of *Ornithodoros*, showed that it was not very closely related to *O. talaje*, a species with which it may have been often confused, but a form which is regarded as new and for it the name *O. coprophilus* n.sp. is proposed.

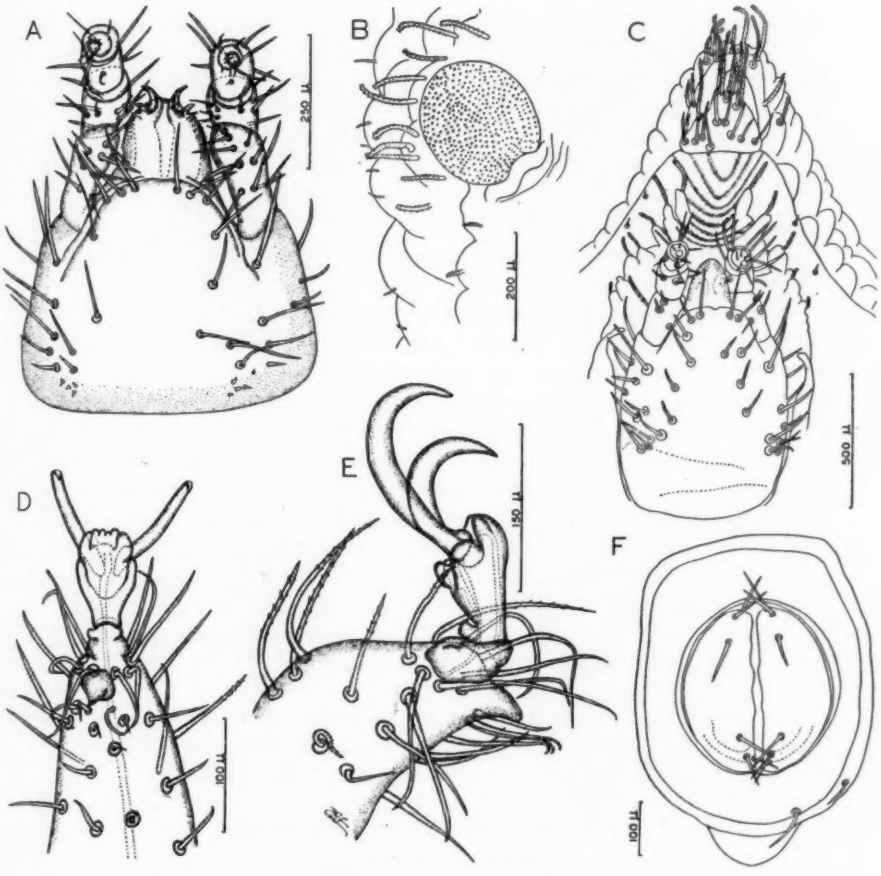
Ornithodorus coprophilus n.sp.

Male. Body piriform, 4 mm. long by 2·57 mm. wide at level of spiracle; light tan in colour, but often appearing darker due to a coating of guano. Integument with numerous mammillae, hairy, especially on legs and palps, dorsal disc absent; two irregular submedian furrows on dorsum. Camerostome without movable "cheeks", but with small finger-like processes. Genital pore between coxae I; anus about midway of distance from posterior margin of body to coxae IV; anal valves with six pairs of hairs arranged as in illustration; a well-marked transverse fold present near posterior border of body. Stigmal plate suboval, 210 by 230μ; ten to twelve conspicuous blunt hairs (text-fig. 2*B*) lateral to stigmal plate. Capitulum conspicuous ventrally, covered dorsally by pointed hood; hypostome small, almost as broad as long,



Text-fig. 1. *Ornithodoros coprophilus* n.sp., male, ventral aspect.

with very small teeth on lateral margins. Legs with coxae contiguous, subequal, II largest, surface smooth; tarsi plain, tapering to well-developed ventral spur.



Text-fig. 2. *Ornithodoros coprophilus* n.sp., A, capitulum of male, ventral aspect; B, right stigmal plate of male and blunt hairs surrounding same; C, anterior end of female, showing capitulum, anterior portion of hood, and finger-like appendages of camerostome, ventral aspect; D, distal portion of right tarsus I of male, ventral aspect; E, distal portion of left tarsus IV of male, lateral aspect; F, anal valves of male showing arrangement of hairs.

Female. Body 5·62 mm. long by 3 mm. wide, proportionately longer than in male; anterior portion pointed, posterior portion somewhat rectangular, lateral margins slightly irregular; deep furrow on each side of dorsum, the furrows converging anteriorly. Integument mammillated, each mammilla with radiating supports about its base and from one to three hairs on its pointed

apex. Camerostome without movable "cheeks", but with five to seven well-developed finger-like appendages projecting laterally and in front of palps, each appendage provided with a short blunt hair. Genital pore at level of interval of coxae I and II; other characters similar to those of male, except that tarsi in female specimens lack well-developed terminal ventral spur.

Nymphs of various sizes resembling adults in appearance. Sexual differentiation possible in last nymphal instar, although genital pore not yet developed.

Holotype. Male, U.S. Nat. Mus. Helm. Coll. No. 40261.

Allotype. Female, U.S. Nat. Mus. Helm. Coll. No. 40262.

Paratypes. Males, females, and nymphs, U.S. Nat. Mus. Helm. Coll. Nos. 40263 and 40264, and U.S. Nat. Mus. Arachnida Coll. No. 1116.

Type host. Unknown, probably bats.

Type locality. Mexico (Linares, Nuevo Leon); also from U.S.A. (Tucson, Arizona).

Remarks. Ornithodoros coprophilus is more closely related to *O. marginatus* Banks 1909–10,[1] a species reported from West Indian bats, than to any of the other species of the genus. The most outstanding differences between these two species are finger-like appendages of the camerostome in *O. coprophilus*, which are absent in *O. marginatus*, and the absence of prominent projections along the margins of the body in *O. coprophilus*, while conspicuous conical projections, each bearing a tuft of hairs, are present along the margins of the body in *O. marginatus*. These two species are undoubtedly more closely related to each other than they are to any other members of the genus. The combination of (1) absence of dorsal disc, (2) plain tarsi, (3) contiguous coxae, and (4) camerostome with finger-like appendages instead of movable "cheeks", are sufficient characters to separate *O. coprophilus* from the other thirty-odd species now recognised as belonging to the genus.

The writer wishes to acknowledge the assistance of Mrs E. B. Fitzgerald, artist of the Bureau of Animal Industry, for aid in the preparations of the drawings accompanying the present description of this tick.

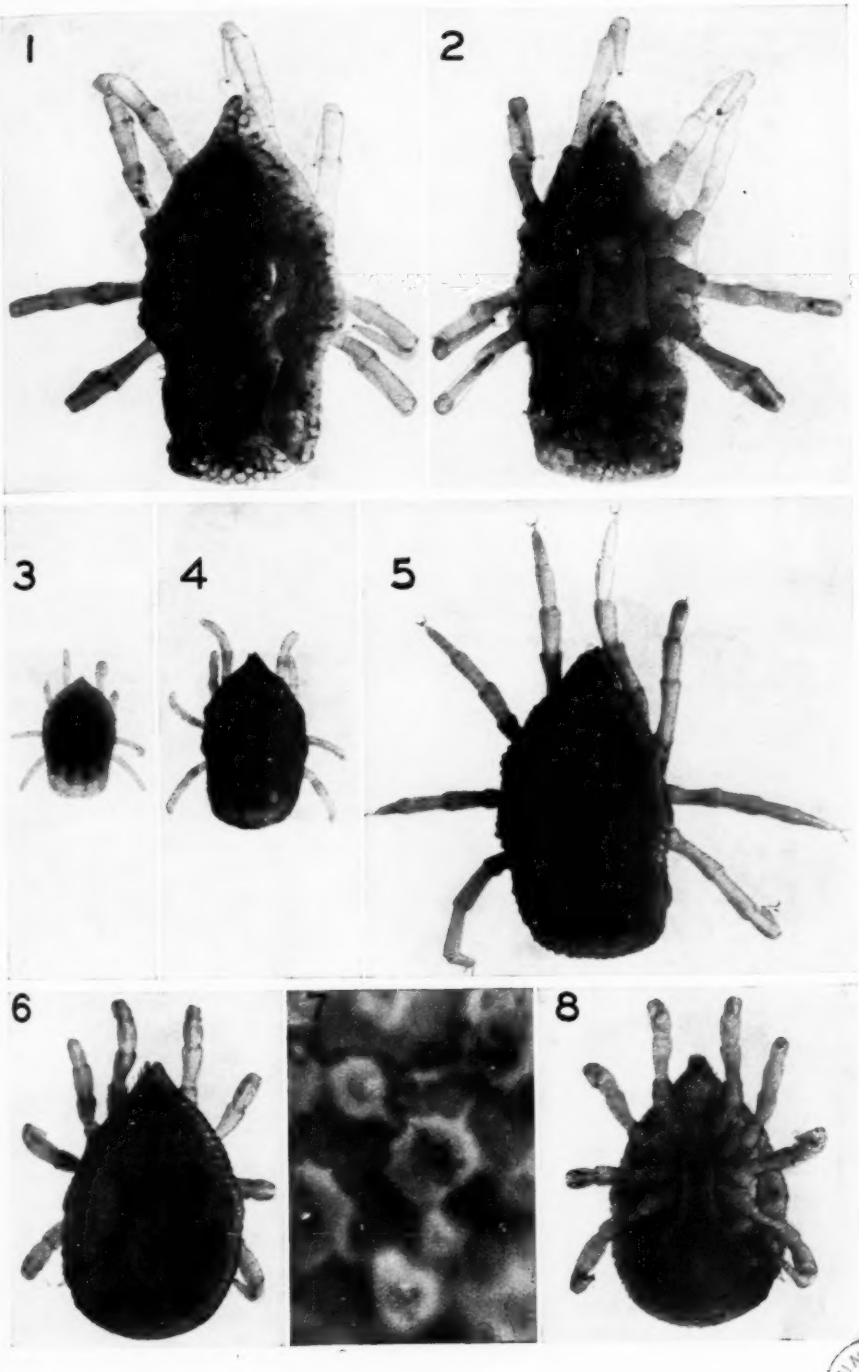
EXPLANATION OF PLATE XVIII

Ornithodoros coprophilus n.sp.

Fig. 1. Female, dorsal aspect. ×10½.
Fig. 2. Female, ventral aspect. ×10½.
Figs. 3–5. Nymphs of various sizes, dorsal aspect. ×10½.
Fig. 6. Male, dorsal aspect. ×10½.
Fig. 7. Mammillae of dorsum of female. ×96.
Fig. 8. Male, ventral aspect. ×10½.

[1] Banks' (*Proc. Ent. Soc. Wash.* **12**, 6, 1910) description was not published until after Hooker (*J. Econ. Ent.* **2**, 415, 1909) in the previous year had inadvertently published Banks' manuscript name, mentioning only the locality.

(*MS. received for publication* 13. III. 1935.—Ed.)



UNIV. OF MICH.

THE PREPARATION OF GRADOCOL MEMBRANES AND THEIR APPLICATION IN THE STUDY OF PLANT VIRUSES

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(With 7 Figures in the Text)

Introductory

Using a method of graded coagulation, W. J. Elford (1931) has evolved a technique for the production of collodion membranes of highly uniform structure. The pores of individual membranes do not vary greatly in size as do those of the acetic acid-collodion membranes previously used, and, in addition, their size is determinable with considerable accuracy. "Gradocol" membranes are in consequence especially suitable for the filtration of viruses and for the estimation of their approximate particle size.

The preparation of these membranes, however, is a process involving the greatest attention to detail, and the technique is a most difficult one for the biologist without any special physico-chemical knowledge. In this paper, therefore, which is the first of a series, it is proposed to describe the difficulties encountered in a study of the membranes during the past three years. It will be advantageous to discuss the technique step by step, showing how errors are liable to arise and how such errors can be eliminated. Full experimental data are given in every case.

Reagents

The ether-alcohol collodion which forms the basis of membranes is the same as that used by Elford. On the whole the uniformity of each batch has been good, though occasionally uneven results were obtained with some samples. The other reagents are all prepared as described in Elford's original paper (1931). Since the slightest trace of water influences the pore size of the membranes it is essential that the reagents should be thoroughly dried, and some difficulty has been experienced in getting them absolutely water-free; this was particularly true of the glacial acetic acid. It has been found advisable to get the latter in small quantities from a reliable chemist who keeps his stock frozen.

The ether-alcohol collodion (Necol) is never kept in the metal container supplied by the maker but is mixed immediately on arrival with equal parts

by weight of acetone and the mixture stored in dried stoppered bottles of 1 litre capacity, thus avoiding errors arising out of ageing or evaporation effects in the necol. These bottles should not be filled more than three parts full to allow for movement of the contents during the shaking process.

It is found advisable to make up the collodion, N 8/40 (1:9) (Elford 1931), in fairly large quantities. "Winchester quart" bottles with screw tops are used for this purpose and filled about three parts full.

It is essential that all glassware used in the preparation of the collodion mixtures should be dry and free of grease. Large vessels are carefully washed in bichromate, dried in a hot-air oven and stored in the constant temperature chamber. Tubes and small glass articles are kept in bichromate-sulphuric acid solution.

Shaking the collodion

In preparing a solution of collodion it is important to ensure that it is homogeneous throughout. As the resultant fluid is viscous it has been found necessary to subject each freshly made solution to at least two hours' shaking on a mechanical shaker. Electrically driven shakers of two types have been used. In one type the bottle of collodion was clamped in a socket at the end of a rotating shaft in such a manner that the axis of the bottle lay at right angles to that of the shaft. It was observed, however, that the fluid tended to collect centrifugally at the ends of the bottle as soon as the machine attained a certain speed. Better results were obtained with a machine which held the bottle horizontally in a wooden cradle and moved it to and fro in line with the axis of the bottle.

The collodion is shaken for two-hour periods after each addition of reagents, so that before a membrane is poured its constituents have had three shakings of two hours each. The first shaking is given when the necol and the acetone are mixed, the second when the parent collodion N 8/40 (1:9) is prepared, and the third after the addition of the acetic acid to produce the desired pore size, just prior to pouring the membrane.

Pouring the membranes

The cell. The cells used for evaporating the collodion solution are each constructed of two square plates of optically ground plate glass. A circular hole was cut in one of these plates, which was then cemented to the other, forming a shallow circular cell. Two of these cells are in use, one having a diameter of 40 cm., the other of 20 cm. The small cell yields seven or eight filtration discs and the large cell thirty to thirty-five.

Levelling the cell. It is absolutely essential in order to secure uniformity of pore size that the plate-glass cell in which the membrane is poured should be perfectly level. For the small cell a hardwood triangle with three levelling screws is used, its sides measuring 22·5 cm.; a similar one with 38 cm. sides is used for the large cell. At first the glass cell was placed on the wooden

surface of the triangle and the whole rested on the adjustable screw feet. In practice this was found to be undesirable, as there was uneven evaporation over an area corresponding to the wooden surface of the triangle where the glass came into contact with it. The triangle was therefore reversed and the cell rested on the adjustable screws while the frame of the triangle rested on the table. This again was found to be a source of error, as the wooden frame tended to rock slightly on the table surface. This difficulty has been overcome by the insertion of three brass rods on which the triangle rests; this gives absolutely rigid support. The final arrangement, then, consists of the glass cell supported on the three brass rods above mentioned. A strong well-seasoned oak table with uralite top carried the whole apparatus (Fig. 1). An ordinary spirit level of good type is used for levelling the cell, and here it may be pointed out that the level should be placed inside the cell and not on the upper glass plate, as the latter is not necessarily in exactly the same plane as the lower plate.

The constant temperature chamber. As the chamber in which the membrane is poured seems to be one of the most important factors in producing uniform membranes it is proposed to discuss it in some detail. The slightest variation in temperature or presence of air currents during the process of setting induces uneven evaporation with consequent variations in average pore size. The chamber used during the first two years was a room, 15 ft. by 9 ft. There were two windows, with close-fitting metal frames; these were covered while the membrane was setting with blinds of brown holland to exclude sunlight. Entrance to the room was by a sliding door running in a groove. The temperature considered most suitable for the membrane setting was that suggested by Elford, 22·5° C. The room was specially equipped with six electric heating units arranged on the wall about 18 in. from the ground at regular intervals round the room. The thermostatic control was at first of the type actuated by the simple expansion of a coiled strip of metal. This was later replaced by a more accurate one of the magnetic type, but with neither of these instruments was it possible to confine the variation in temperature to within a range of less than two or three degrees above or below the mean. All the apparatus was put ready and the current switched on two hours before the membrane was poured. It was found in practice that the temperature varied from 20 to 25° C., the rise being due to the fact that the heating units retained their heat for a considerable time after the current was cut off. Every effort was made to exclude draughts and air currents and the room was kept closed during the setting period. From data obtained for membranes poured under these conditions, it was found that the percentage error in average pore diameter (A.P.D.) was very large. This was considered to be due to uneven evaporation caused by the fluctuations in temperature and possibly by air currents. Needless to say the variations in A.P.D. of the membranes were reflected in the uneven results obtained in filtrations with various plant viruses.

The old chamber was therefore discarded and another one specially built.

This chamber measures 9 ft. high by 3 ft. by 3 ft. and has double cavity walls 2 in. thick made of "Beaver" board. The front of the chamber for a height of 6 ft. 6 in. opens outward to allow access to the interior, but this is used only when special adjustments inside the chamber are necessary. All the operations of pouring the membranes can be carried out by means of a small

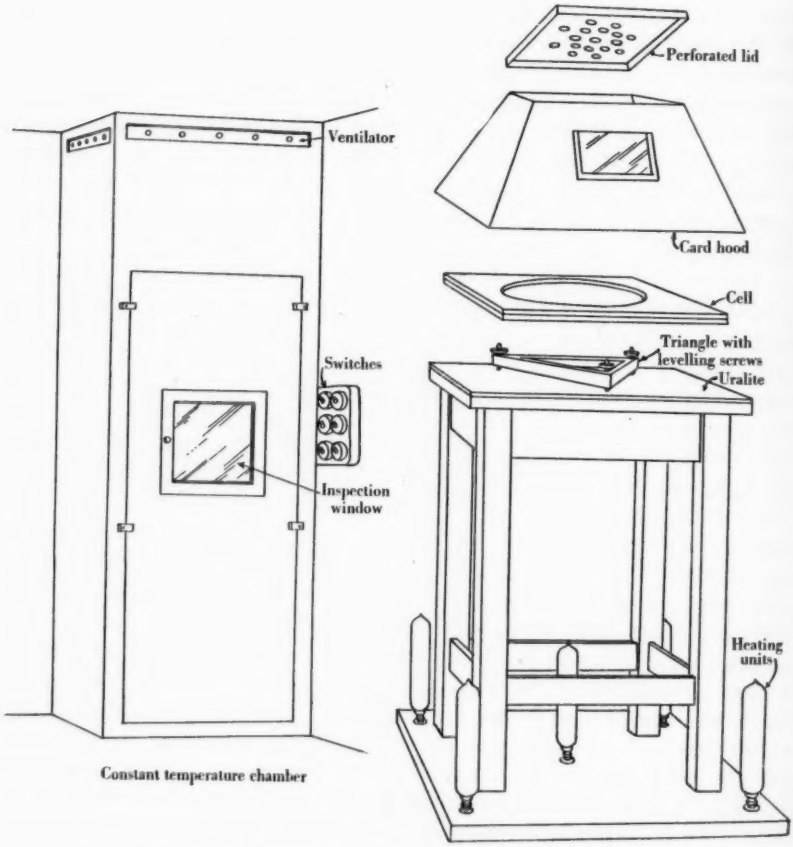

Fig. 1. Details of constant temperature chamber, and apparatus used for evaporation of membranes.

door let into the larger one, 3 ft. 6 in. from the ground (Fig. 1). This small door is partly of glass to allow observations to be made during the evaporation process. Adjustable ventilators are fitted at the top of the chamber on two sides to allow for the escape of the amyl alcohol vapour, etc., after evaporation has ceased. All joints are covered over with beading to exclude air currents. The heating units consist of five candle lamps controlled by a mercury-toluol

thermostat. The lamps are fixed to a square wooden frame on the floor, one lamp at each corner and one in the centre. The legs of the table carrying the glass cell also fit into the wooden frame in the manner shown in the figure. This arrangement has been adopted as the one giving the most uniform heating. A bulb is arranged in such a way that the light falls on the surface of the membrane and all switches are outside so that the electrical apparatus can be controlled without opening the chamber. The use of the lamp in the centre, *i.e.* under the table, was later discontinued for fear of uneven heating. The thermostat is set to give a temperature of 22·5° C., and this has been maintained without more than half a degree variation. The chamber was built in a corner of the laboratory on the top floor of the Molteno Institute. During the summer, however, the temperature in the laboratory and consequently in the chamber rose to 25° C. and over, too high for satisfactory setting of the membranes. A duplicate chamber for use in summer was therefore constructed in the basement, where the temperature seldom exceeded 19° C. even in the hottest weather. This second chamber is similar to the first except that it has double cavity walls of wood instead of "Beaver" board, and measured 6 ft. 6 in. by 4 ft. by 4 ft.

Humidity. Readings of the humidity in the chamber were taken by means of a recording hygrometer kindly lent by the Low Temperature Research Station. The record given on the chart was practically a straight line, the humidity being 55 per cent. There was very little alteration in the humidity while the membrane was setting in the chamber.

Evaporation. During the setting of the membrane a paper or cardboard hood is placed over the cell to prevent air currents and to control evaporation. As Elford has shown (1931) the finer grades of variation in A.P.D. can be obtained by varying the evaporation rates, and the writers have found that the type of hood used influences the rate of evaporation and consequent pore size to a high degree. Various types of hoods and collars have been used, the one finally selected being made of card, cut to fit exactly round the outside of the cell and fitted with a glass window back and front which allows observation of the progress of the membrane during the evaporation period (see Fig. 1).

Flooding the membrane. At the end of the evaporation period the action of the solvents is stopped by flooding the cell with distilled water which has been kept in the chamber in a stoppered bottle. This operation must be carried out with care to ensure even and rapid flooding of the membrane. At first the practice was to pour the water rapidly into the centre of the cell; owing, however, to the possibility that a slight depression might be caused in the middle of the membrane by this procedure, water was run over the membrane surface by a simultaneous flooding from the sides with water from two 500 c.c. beakers. There are one or two points of procedure after the membrane has been flooded which may appropriately be emphasised here. When the 20 cm. cell is used the membrane is allowed to remain *in situ* for about two hours

after flooding. By that time it has shrunk slightly away from the sides of the cell and can be lifted out and placed bodily in a large crystallising dish. The membrane is given the requisite fourteen days' washing in this dish and not cut up into discs until the end of that period. The large 40-cm. membrane is removed from the cell as soon as it has shrunk away from the edges, and cut up into quarters with a pair of scissors or safety razor blade for subsequent washing, as otherwise it would be too large for convenient handling. Alternatively it is cut up at once with the punch into discs and washed in that form. For washing the discs, about thirty-six in number from a large membrane, it is found convenient to use deep Petri dishes 10 cm. by 5 cm. These, however, as explained in a subsequent paragraph, are unsuitable for storing the discs after sterilisation.

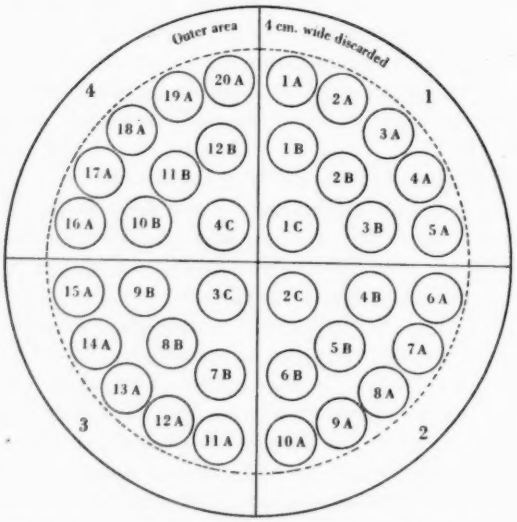

Fig. 2. Diagram of membrane showing convention used in numbering discs.

The washing process is continued for fourteen days as recommended by Elford (1931), with two changes of distilled water daily. An experiment was performed to examine the stability of the membrane during the washing. Six discs taken from a large membrane were standardised at four-day intervals throughout the washing period commencing three days after the actual setting of the membrane, only the time of flow of water (T.F.W.) being measured. Very little change was apparent during the sixteen days of the test, the T.F.W. values remaining constant for individual membrane discs.

Cutting the discs. The membrane sheet is placed on a piece of plate glass and the discs are cut from it with a steel punch of standard size. Each disc is marked by means of a pencilled number at the extreme edge. The discs are numbered according to the convention shown in Fig. 2.

Sterilisation of membrane discs. Since autoclaving is too drastic a method of sterilisation (Elford, 1931), the discs have to be steamed for two hours in an ordinary steamer at 100° C. For this purpose they are placed in distilled water in small glass vessels with well-fitting ground-glass stoppers. These vessels are similar to the ordinary dipping bottles used for microscope slides and of the same diameter but only half the height, measuring 5 cm. in depth by 5 cm. in width exclusive of a flange at top and bottom. Petri dishes were found unsuitable for storing membranes after sterilisation, as moulds and bacteria were able to gain access by the loosely fitting lids.

In order to investigate the effect of steaming on verage pore size under the writers' conditions, a series of membranes was caubrated before and after steaming. The results compare favourably with Elford's findings of a 5 per cent. difference due to steaming.

CALIBRATION OF THE MEMBRANES

The A.P.D. of the membranes is calculated from the rate of flow of water data and a determination of the water content of each particular grade of membrane. The formula used is based upon the assumption that Poiseuille's law governs the rate of flow of water through the pores of the membranes, which for this purpose must be considered as a number of capillary tubes running at right angles to the membrane surface:

$$r = 2l\frac{2q\eta}{pv},$$

where r = radius of pores in cm.

l = length of capillary = thickness of membrane in cm.

q = volume of water passing in c.c./sec.

η = viscosity of water at temperature of measurement in C.G.S. units.

p = pressure producing flow in dynes/sq. cm.

v = total volume of pores (assumed equivalent to water content of membrane) (Elford, 1931).

There are therefore three values to be obtained for each membrane before its A.P.D. can be determined; these are thickness, water content and the rate of flow of water value.

Thickness of membrane discs. At first the thickness of the discs was measured with an ordinary screw micrometer gauge. In its use, however, there lies a possible source of error arising from the personal factor. In other words it is not possible always to use the same pressure and, with a plastic material like collodion, uniform results are not obtainable. The screw micrometer was therefore discarded and a more accurate and elaborate piece of apparatus was employed. This is illustrated in Fig. 3. It was obtained, mounted in the manner shown, from W. G. Pye and Co., Cambridge.

Micrometer. The micrometer gauge illustrated in Fig. 3 is of the direct-reading dial type mounted on a suitable stand. The pin (*A*) is raised, the membrane disc placed on the table (*B*) and the pin released. In order that the end of the pin shall not depress the surface of the membrane and give a false reading, a small plate of optically ground glass, or thin cover-glass (No. 0) of known thickness is placed on the membrane disc before the pin is lowered. The combined thickness is then read off directly from the scale on the dial (in this case calibrated in 0·01 mm.), and that of the glass plate subtracted. Adjustments are provided for raising and lowering the table and the micrometer.

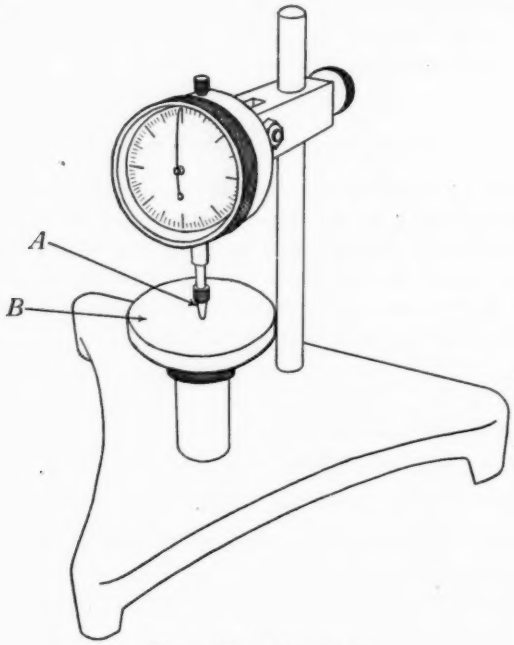

Fig. 3. Micrometer gauge.

The membrane disc is placed under the glass plate and several readings are taken at various points on the disc. In preparing membranes of good uniformity it is most important that the thickness should not vary more than 0·05 mm., any unevenness in evaporation being reflected in the variations of the subsequent thickness.

Water content. The water content is assumed to be the difference between the wet and dry weights of the membrane discs. Here again it has been found best to adopt a uniform mode of procedure. Before taking the wet weight of the discs excess of moisture is removed by placing them between two hard filter papers and subjecting them to a standard pressure. They are then care-

fully weighed, being covered during the process with a watch glass to prevent continual loss of weight by evaporation. The drying oven used is an ordinary Hearson's gas incubator with a capsule thermostat, the temperature being set at 70° C. The discs are allowed to remain in this oven for at least four hours.

From the appearance of the dried membrane it is possible to gauge roughly the approximate degree of permeability, porous membranes appear white and opaque while impermeable discs show a bluish transparency.

After this paper had been written, Elford and Ferry (1935) published some further work upon the calibration of graded collodion membranes in which they give a formula for calculating the specific water content. This value is defined as the volume of water contained in 1 c.c. of membrane when the pores are full of water, as is the case for a disc washed and equilibrated in distilled water, thus:

$$S = \frac{W_w - W_d}{A_t},$$

where S = specific water content.

W_w = weight of membrane, pores full of water.

W_d = weight of dry membrane.

A = area of membrane cross-section.

t = membrane thickness.

The specific water content values for each disc have therefore been inserted in Table I.

Rate of flow of water values (R.F.W.). It is in obtaining consistent and uniform values for the time of flow of water through the different discs of the same membrane that the writers have experienced the greatest difficulty, although the values for individual discs have been constant enough. In this phase of the work more than any other it is necessary for the distilled water to be clean and the glassware absolutely free of grease. It is also recommended that the rubber stoppers in the rate-of-flow-of-water apparatus be coated with paraffin wax to prevent small pieces of rubber from becoming detached and interfering with even rate of flow. The initial difficulty experienced was the slight but persistent increase in the time of flow of water in successive readings for the same membrane disc. The increase was apparently due to the gradual plugging of the membrane pores by impurities in the water and grease from the glassware and was eliminated after careful attention to these points. The difficulty however is still liable to recur, at all events under the writers' conditions.

R.F.W. apparatus. Three types of rate of flow of water apparatus have been used and they are illustrated in Figs. 4 and 5. The apparatus shown in Fig. 4 is designed by Elford and that in Fig. 5 is a modification of it. Fig. 5, diagram 3, shows an adaptation of the apparatus used by Bauer and Hughes (1934). This is made of nickel-plated brass and has the advantage that it can be screwed tightly without fear of breakage when used under high pressures. It has, however, the disadvantage that air bubbles may lodge unseen in the

metal chamber. The following point has been found important in ensuring uniform data. Slight variation in room temperature during the time of flow of water tests was reflected in the results obtained. It was therefore deemed advisable to immerse the disc and ebonite cell in a bath of distilled water kept at a constant temperature of 20° C.

The apparatus illustrated in Fig. 5 is a modification by MacClement and the writers of that designed by Elford and shown in Fig. 4. The membrane disc is held between the ground flanges of two short open-ended glass tubes, which in turn are held between two ebonite plates clamped with bolts and

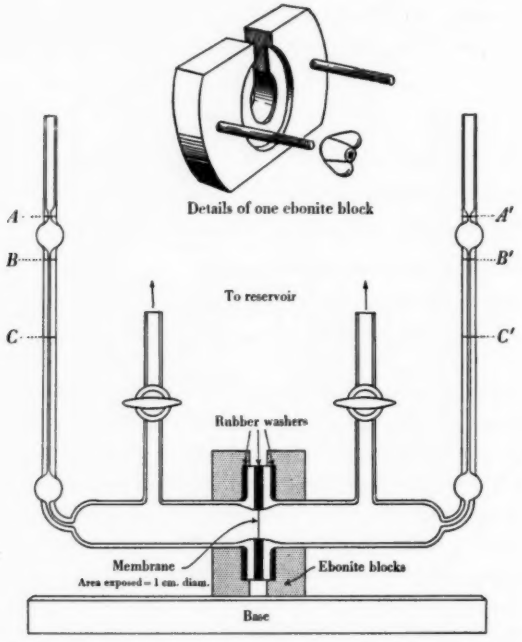

Fig. 4. Elford's apparatus for measuring rate of flow of water.

wing-nuts. The bolts and ebonite plates are hinged and so arranged that the two halves may be easily separated to give free access for insertion and removal of the discs (see Fig. 5, diagram 2). The flanged tubes, which together form the glass cell, are plugged at their outer ends by rubber bungs, bored to admit the ends of the calibrated tubes with which the measurements are made. These are arranged vertically on either side of the cell as shown in Fig. 5, diagram 1, and can be removed and replaced by others of different calibrations without dismantling the apparatus. Water reservoirs, connected to the bases of the calibrated tubes, are provided for rapid filling of the latter, and air pressure from an aspirator is controlled by two three-way taps as

illustrated. The whole apparatus is mounted on a board so arranged that it can be readily lifted clear of the water-bath in which the cell is normally immersed (see Fig. 5, diagram 4).

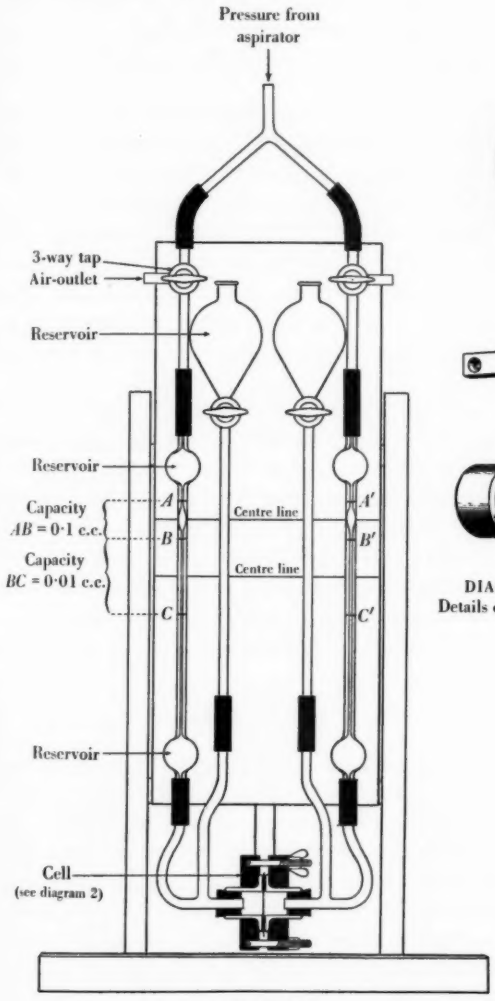

DIAGRAM 1

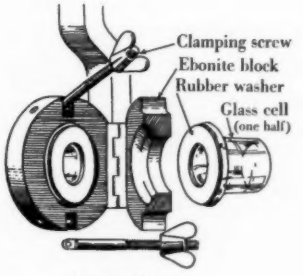

DIAGRAM 2
Details of ebonite cell

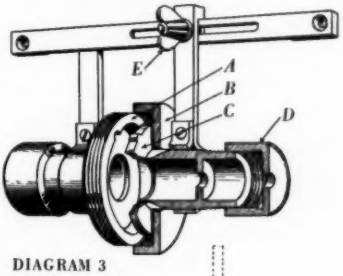

DIAGRAM 3
Details of metal cell

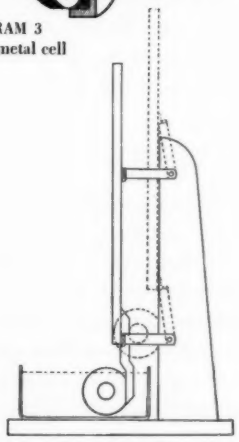

DIAGRAM 4

Fig. 5. Details of other types of apparatus used for measuring rate of flow of water.

Procedure. When the quantity of water to be run through has been determined, according to the permeability of the membrane, tubes of suitable calibration are fitted. Those illustrated are constructed for quantities of 0·1

and 0·01 c.c. Assuming that 0·1 c.c. is the required quantity, then the left-hand tube is filled from its reservoir to the mark *A* and the right-hand tube to *B'*. Pressure is applied to the left-hand tube, and the air outlet to the right-hand is open. The time taken for the column of water to fall from *A* to *B* is recorded by means of a stop-watch. Pressure is then changed over to the right and the flow timed from *A'* to *B'*. This process is repeated until six readings have been taken.

The metal cell figured in Fig. 5, diagram 3, is similar to that described by Bauer and Hughes (1934). It is constructed of nickel plated brass and embodies the same principles as the ebonite and glass cell above described. The membrane is held securely in a socket enclosed by the screwed rim *A* (see Fig. 5, diagram 3) into which fits the flange *C*, the two being clamped together by the knurled ring *B*. At either end of the cell is a screw cap *D* which compresses a rubber bung tightly round the end of the glass calibrated tube. The two halves of the cell are suspended by brass supports, one of which is movable to allow access to the membrane, and may be clamped in position by the nut *E*.

Filtration. In the earlier part of this work both negative and positive pressure were used for filtration. For negative pressure a glass filter of German design, Der Buwar Apparat, was employed. Later, the negative pressure apparatus was discarded and all filtrations are now carried out with Elford's ultra filter (Barnard and Elford, 1931) under positive pressure. A simple pressure distributor is used by means of which it is possible to carry out four or five filtrations simultaneously. The chief disadvantages of negative pressure were found to be the inconvenience of handling for fractional filtration, insufficient pressure and excessive foaming of the filtrate.

Variations in A.P.D. of membranes

During the first part of this work the membranes showed a mean variation in A.P.D. in the order of 20–35 per cent. At the beginning it was only considered necessary to standardise one or two discs out of each membrane, the assumption being that the rest of the discs were uniform with them, for under optimum conditions this would be so. The point is, however, that standardising one or possibly two discs did not give sufficient indication of the wide variation which was actually existing under the experimental conditions at the time. It was only after the failure to obtain consistent filtration results with a series of viruses that the rather arduous work of standardising the entire membrane was undertaken. The variations in A.P.D. then became apparent, the filtration of the viruses was discontinued for the time being and the various experiments here described were instituted in an endeavour to find where the faults of procedure lay.

At the suggestion of Dr Elford three membranes were poured under identical conditions using the same parent collodion. This was carried out in the

new constant temperature chamber with an N 8/40 (1:9) system. The results now show a certain uniformity not hitherto manifested. Although there is still a variation in T.F.W. values of 10–15 per cent. from the mean they rise continuously to a maximum and fall again when examined in relation to the numbers of the membrane discs.

These numbers, as already explained, indicate the relative position of the latter in a circle around a central disc. The distribution of the porosities shows that one-half of the membrane differed from the other, and this may have been due either to faulty levelling of the cell or uneven evaporation and one-sided temperature effects.

These points and the other sources of error outlined in this paper have been investigated and membranes can now be prepared in which the variation of A.P.D. may be as low as 5 per cent. Membranes showing a variation in excess

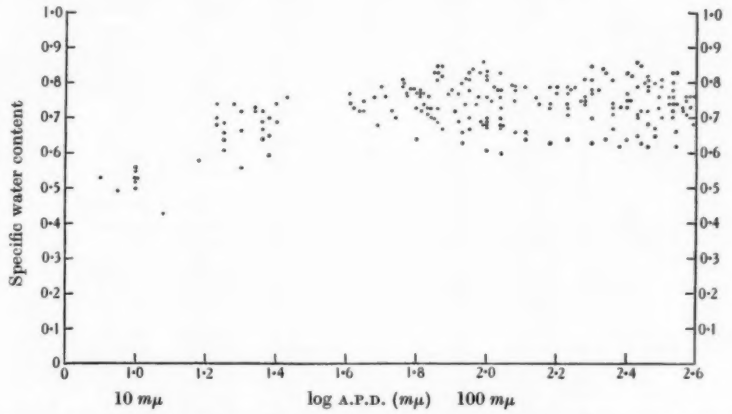

Fig. 6. Graph showing relationship between water contents and porosities of membranes.

of 15 per cent. error continue to occur at intervals, and the reason for this is not known. Such membranes are always discarded. The data of a representative series of membranes as prepared under the writers' present conditions are given in Table I, and a graph showing the relationship between the specific water contents and the porosities of a large number of discs is shown in Fig. 6.

PREPARATION OF PLANT VIRUS SUSPENSION FOR FILTRATION

There is little doubt that the plant-virus worker encounters a serious difficulty in the way of a successful application of this membrane technique which is not met with in the same degree by the animal-virus worker. The difficulty lies in the nature of the plant extract in which the virus must be filtered. This material, which is usually at an acid *p*H varying from 5·8 to 6·6, contains many proteins which are easily precipitated together with tarry

Table I. *Data of twenty-two membranes of differing systems and of* A.P.D. *varying from* 1 μ *to* 10 $m\mu$*: the table shows the degree of uniformity obtained and the mean error in each case.*

System	Disc no.	T.F.W. sec.	Pressure cm. H_2O	Volume c.c.	Thickness cm.	Wet weight g.	Dry weight g.	Specific water content g.	A.P.D. μ
N 8/40 (1 : 9) + 0·6 % HAc	1	45	14·8	2·1	0·016	0·175	0·047	0·7053	1·07
Mean A.P.D. = 1·04 μ	2	48	14·8	2·1	0·016	0·167	0·045	0·6723	1·06
Error = 8 %	3	47	14·8	2·1	0·0155	0·165	0·046	0·6800	1·05
	4	48	14·8	2·1	0·0155	0·163	0·045	0·6713	1·05
	5	55	14·8	2·1	0·015	0·177	0·047	0·7640	0·96
N 8/40 (1 : 9) + 0·5 % HAc	1	46	14·8	2·1	0·0135	0·155	0·046	0·7119	0·974
Mean A.P.D. = 0·97 μ	2	45	14·8	2·1	0·0130	0·151	0·045	0·7190	0·961
Error = 3 %	3	44	14·8	2·1	0·0135	0·160	0·048	0·7314	0·980
	4	42	14·8	2·1	0·0130	0·158	0·048	0·7461	0·977
	5	44	14·8	2·1	0·0140	0·167	0·050	0·7369	0·996
N 8/40 (1 : 9) + 0·7 % HAc	1	92	14·8	2·1	0·0170	0·183	0·046	0·7106	0·772
Mean A.P.D. = 0·73 μ	2	67	14·8	2·1	0·0165	0·182	0·047	0·7213	0·88
Error = 20 %	3	118	14·8	2·1	0·0170	0·186	0·047	0·7209	0·678
	4	117	14·8	2·1	0·0180	0·190	0·046	0·7053	0·708
	5	124	14·8	2·1	0·0180	0·183	0·046	0·6710	0·705
	6	114	14·8	2·1	0·0170	0·185	0·046	0·7209	0·689
N 8/40 (1 : 9) + 0·8 % HAc	1	8·5	29·0	0·2	0·0160	0·1890	0·0522	0·7539	0·56
Mean A.P.D. = 0·56 μ	2	9·1	29·0	0·2	0·0160	0·1736	0·0490	0·6866	0·57
Error = 16 %	3	12·4	29·0	0·2	0·0160	0·1810	0·0490	0·7274	0·47
	4	6·4	29·0	0·2	0·0160	0·1825	0·0490	0·7357	0·65
	5	9·1	29·0	0·2	0·0150	0·1664	0·0470	0·7018	0·55
	6	7·4	29·0	0·2	0·0150	0·1712	0·0460	0·7358	0·59
N 8/40 (1 : 9) + 1·4 % HAc	1	8·0	60·2	0·2	0·0160	0·1224	0·0494	0·4023	0·55
Mean A.P.D. = 0·50 μ	2	7·9	60·2	0·2	0·0150	0·1216	0·0476	0·4349	0·51
Error = 12 %	3	15·1	60·2	0·2	0·0160	0·1030	0·0436	0·3273	0·44
	4	10·6	60·2	0·2	0·0150	0·1130	0·0454	0·3973	0·47
	5	8·2	60·2	0·2	0·0160	0·1273	0·0482	0·4359	0·52
N 8/40 (1 : 9) + 1·5 % HAc	1	54·8	65·8	2·1	0·0155	0·1786	0·0480	0·7427	0·44
Mean A.P.D. = 0·45 μ	2	54·9	65·8	2·1	0·0165	0·1938	0·0480	0·7791	0·447
Error = 11 %	3	54·2	65·8	2·1	0·0165	0·1895	0·0470	0·7614	0·45
	4	44·0	65·8	2·1	0·0155	0·1754	0·0480	0·7247	0·50
	5	56·5	65·8	2·1	0·0155	0·1752	0·0470	0·7293	0·44
	6	51·8	65·8	2·1	0·0150	0·1756	0·0480	0·7500	0·44
N 8/40 (1 : 9) + 1 % HAc	1	54·5	65·0	2·1	0·0170	0·1813	0·0470	0·6966	0·48
Mean A.P.D. = 0·415 μ	2	90·1	65·0	2·1	0·0190	0·1958	0·0480	0·6858	0·40
Error = 17 %	3	58·6	65·0	2·1	0·0170	0·1908	0·0456	0·7532	0·45
	4	75·9	65·0	2·1	0·0170	0·1844	0·0455	0·7206	0·40
	5	89·9	65·0	2·1	0·0180	0·1946	0·0480	0·7181	0·38
	6	94·7	65·0	2·1	0·0180	0·1860	0·0464	0·6837	0·38
N 8/40 (1 : 9) + 0·6 % HAc	1	116·5	62·9	2·1	0·020	0·2148	0·0500	0·7264	0·36
Mean A.P.D. = 0·35 μ	2	119·5	62·8	2·1	0·020	0·2280	0·0510	0·7812	0·34
Error = 11 %	3	143·1	62·8	2·1	0·020	0·2240	0·0505	0·7647	0·31
	4	93·8	62·9	2·1	0·020	—	—	*0·7626	0·39
	5	103·0	62·9	2·1	0·020	—	—	*0·7626	0·37
	6	99·2	62·9	2·1	0·020	—	—	*0·7626	0·38
	7	128·7	62·9	2·1	0·020	—	—	*0·7626	0·33
N 8/40 (1 : 9) + 1·2 % HAc	1	50·2	60·7	0·2	0·0160	0·1835	0·0434	—	(0·17)
Mean A.P.D. = 0·31 μ	2	16·7	60·7	0·2	0·0160	0·1879	0·0444	0·7907	0·29
Error = 13 %	3	18·15	60·7	0·2	0·0180	0·2071	0·0486	0·7764	0·30
	4	17·4	60·7	0·2	0·0150	0·1780	0·0434	0·7911	0·27
	5	14·1	60·7	0·2	0·0160	0·1880	0·0480	0·7714	0·32
	6	11·0	60·7	0·2	0·0155	0·1821	0·0440	0·7856	0·35
N 8/40 (1 : 9) + 1·6 % HAc	1	26·7	65·8	0·2	0·0195	0·1832	0·0446	0·6267	0·24
Mean A.P.D. = 0·20 μ	2	36·6	66·3	0·2	0·0195	0·1884	0·0424	0·6621	0·20
Error = 20 %	3	38·2	66·3	0·2	0·020	0·2024	0·0422	0·7062	0·19
	4	44·7	66·3	0·2	0·0185	0·1992	0·0410	0·7541	0·16
	5	34·5	65·8	0·2	0·0195	0·1916	0·0430	0·6720	0·20
	6	43·6	66·3	0·2	0·0195	0·1972	0·0416	0·7036	0·18
N 8/40 (1 : 9) + 1·8 % HAc	1	59·2	65·8	0·2	0·0170	0·1940	0·0468	0·7635	0·14
Mean A.P.D. = 0·16 μ	2	40·9	65·8	0·2	0·0180	0·2088	0·0507	0·7745	0·17
Error = 12·5 %	3	49·8	65·8	0·2	0·0170	0·1912	0·0494	0·7353	0·15
	4	41·2	66·0	0·2	0·0170	0·1896	0·0478	0·7353	0·17
	5	42·7	66·0	0·2	0·0170	0·1972	0·0490	0·7688	0·16
	6	47·4	66·0	0·2	0·0170	0·2007	0·0490	0·7868	0·15

* Average values.

Table I (*continued*)

System	Disc no.	T.F.W. sec.	Pressure cm. H_2O	Volume c.c.	Thickness cm.	Wet weight g.	Dry weight g.	Specific water content g.	A.P.D. μ
N 8/40 (1 : 9) + 1·8 % HAc	1	118·5	65·6	0·2	0·0195	0·1980	0·0410	0·7050	0·11
Mean A.P.D. = 0·10 μ	2	167·6	65·6	0·2	0·0195	0·1974	0·0420	0·6977	0·09
Error = 10 %	3	102·4	65·6	0·2	0·020	0·1974	0·0444	0·6745	0·11
	4	123·8	65·6	0·2	0·020	0·1960	0·0418	0·6798	0·11
	5	139·6	65·6	0·2	0·020	0·2040	0·0430	0·7098	0·10
	6	144·5	65·6	0·2	0·020	0·1950	0·0418	0·6754	0·10
N 8/40 (1 : 9) + 2·5 % HAc	1	238·9	51·3	0·2	0·023	0·2440	0·0454	0·7614	0·095
Mean A.P.D. = 0·094 μ	2	171·7	51·3	0·2	0·023	0·2522	0·0480	0·7827	0·111
Error = 21 %	3	312·8	51·0	0·2	0·020	0·2126	0·0455	0·7367	0·074
	4	179·0	51·3	0·2	0·023	0·2440	0·0454	0·7614	0·111
	5	258·8	51·3	0·2	0·022	0·2396	0·0452	0·7791	0·088
	6	251·3	51·0	0·2	0·021	0·2280	0·0450	0·7685	0·083
N 8/40 (1 : 9) + 2 % HAc	1	263·6	51·0	0·2	0·020	0·2118	0·0453	0·7340	0·081
Mean A.P.D. = 0·08 μ	2	302·7	52·0	0·2	0·0215	0·2222	0·0494	0·7086	0·079
Error = 17·5 %	3	308·1	51·0	0·2	0·0215	(0·2982)	0·0494	(1·021)	0·066
	4	288·9	51·3	0·2	0·022	0·2027	0·0470	0·6419	0·087
N 8/40 (1 : 9) + 1 % HAc	1A	156·6	91·5	0·2	0·0145	0·1514	0·0414	0·6688	0·075
Mean A.P.D. = 0·072 μ	6A	159·5	91·9	0·2	0·0140	0·1528	0·0412	0·7028	0·071
Error = 11 %	11A	154·3	91·9	0·2	0·0140	0·1588	0·0424	0·7331	0·070
	16A	109·2	91·9	0·2	0·0150	0·1590	0·0442	0·6747	0·090
	2B	157·6	92·3	0·2	0·0135	0·1490	0·0410	0·7053	0·070
	5B	180·3	91·5	0·2	0·0135	0·1514	0·0400	0·7274	0·064
	8B	162·3	91·9	0·2	0·0135	0·1530	0·0410	0·7314	0·068
	11B	145·1	91·9	0·2	0·0140	0·1550	0·0412	0·7103	0·073
	2C	167·7	91·5	0·2	0·0130	0·1452	0·0386	0·7213	0·066
N 8/40 (1 : 9) + 1 % HAc	1A	304·0	91·5	0·2	0·0155	0·1816	0·0430	0·7884	0·051
Mean A.P.D. = 0·060 μ	6A	232·5	91·5	0·2	0·0155	0·1756	0·0416	0·7623	0·059
Error = 15 %	11A	236·5	91·5	0·2	0·0160	0·1882	0·0436	0·7969	0·058
	16A	219·0	91·5	0·2	0·0170	0·1908	0·0442	0·7605	0·064
	2B	234·5	91·5	0·2	0·0150	0·1744	0·0410	0·7900	0·057
	5B	230·6	91·5	0·2	0·0150	0·1778	0·0412	0·8028	0·057
	8B	213·3	91·5	0·2	0·0160	0·1836	0·0420	0·7802	0·062
	11B	219·5	91·5	0·2	0·0165	0·1782	0·0424	0·7256	0·065
	2C	217·6	91·5	0·2	0·0160	0·1732	0·0410	0·7285	0·064
N 8/40 (1 : 9) + 1 % HAc	6A	295·0	90·4	0·2	0·0209	—	—	—	0·050
Mean A.P.D. = 0·057 μ	8A	305·4	90·4	0·2	0·0202	—	—	—	0·053
Error = 12 %	10A	310·8	90·4	0·2	0·0200	—	—	—	0·052
	16A	290·3	90·9	0·2	0·0220	—	—	—	0·059
	18A	307·1	90·9	0·2	0·0219	—	—	—	0·057
	20A	281·7	90·9	0·2	0·0211	—	—	—	0·057
	4B	260·2	90·9	0·2	0·0211	—	—	—	0·060
	5B	273·1	90·9	0·2	0·0208	—	—	—	0·057
	6B	273·6	90·9	0·2	0·0207	—	—	—	0·057
	10B	257·0	90·9	0·2	0·0222	—	—	—	0·063
	11B	254·5	90·9	0·2	0·0221	—	—	—	0·063
	12B	259·3	90·9	0·2	0·0218	—	—	—	0·062
	7A	—	—	—	0·0206	0·2490	0·0437	0·8786	—
	9A	—	—	—	0·0203	0·2566	0·0436	0·9251	—
	17A	—	—	—	0·0220	0·2766	0·0465	0·9241	—
	19A	—	—	—	0·0216	0·2700	0·0460	0·9141	—
	2C	—	—	—	0·0212	0·2662	0·0440	0·9243	—
	4C	—	—	—	0·0216	0·2662	0·0438	0·9078	—
N 8/40 (1 : 9) + 3 % HAc	1	506·0	90·6	0·2	0·0180	0·1916	0·0436	0·7249	0·045
Mean A.P.D. = 0·049 μ	2	378·1	90·6	0·2	0·0190	0·2078	0·0440	0·7600	0·052
Error = 8 %	3	441·7	90·6	0·2	0·0185	0·1862	0·0430	0·6776	0·049
N 8/40 (1 : 9) + 8 % HAc	1	130·9	91·4	0·0377	0·0185	0·2008	0·0466	0·7695	0·041
Mean A.P.D. = 0·041 μ	2	126·3	91·4	0·0377	0·0180	0·1982	0·0464	0·7433	0·041
Error = 2 %	3	130·7	91·4	0·0377	0·0190	0·2054	0·0484	0·7285	0·042
N 8/40 (1 : 9) + 2 % HAc	1	29·1	91·0	0·0081	0·0130	0·1488	0·0432	0·7163	0·023
Mean A.P.D. = 0·023 μ	2	37·4	91·0	0·0081	0·0120	0·1388	0·0434	0·6642	0·020
Error = 17 %	3	27·5	91·4	0·0081	0·0150	0·1678	0·0425	0·7364	0·025
	4	27·4	91·0	0·0081	0·0135	0·1478	0·0410	0·6979	0·024
	5	29·6	91·4	0·0081	0·0125	0·1444	0·0424	0·7223	0·022
	6	21·8	91·4	0·0081	0·0150	0·1688	0·0402	0·7560	0·027

Table I (*continued*).

System	Disc no.	T.F.W. sec.	Pressure cm. H_2O	Volume c.c.	Thickness cm.	Wet weight g.	Dry weight g.	Specific water content g.	A.P.D. μ
N 8/40 (1 : 9) +1 % HAc	1A	462·3	91·5	0·2	0·0090	0·1152	0·0456	0·6819	0·017
Mean A.P.D. = 0·018 μ	6A	459·2	90·3	0·2	0·0090	0·1072	0·0444	0·6153	0·018
Error =17 %	11A	682·2	91·3	0·2	0·0090	0·1020	0·0432	0·5761	0·015
	16A	415·0	91·3	0·2	0·0090	0·1012	0·0444	0·5564	0·020
	2B	352·6	91·5	0·2	0·0080	0·1026	0·0430	0·7375	0·019
	5B	417·1	91·5	0·2	0·0085	0·1042	0·0430	0·6649	0·018
	8B	453·0	90·3	0·2	0·0085	0·1010	0·0422	0·6389	0·018
	11B	401·4	91·5	0·2	0·0085	0·1064	0·0432	0·6866	0·018
	2C	407·5	91·5	0·2	0·0080	0·1022	0·0422	0·7427	0·017
N 8/40 (1 : 9) +1 % HAc	1A	575	91·5	0·1	0·0077	0·0812	0·0434	0·4328	0·012
Mean A.P.D. = 0·010 μ	6A	600	91·5	0·1	0·0070	0·0851	0·0430	0·5303	0·010
Error =20 %	11A	865	91·5	0·1	0·0060	0·0780	0·0418	0·5319	0·008
	16A	680	91·5	0·1	0·0073	0·0880	0·0440	0·5315	0·010
	2B	522	91·5	0·1	0·0062	0·0798	0·0410	0·5517	0·010
	5B	577	91·5	0·1	0·0060	0·0759	0·0404	0·5240	0·010
	8B	814	91·5	0·1	0·0060	0·0743	0·0406	0·4951	0·009
	11B	566	91·5	0·1	0·0062	0·0816	0·0420	0·5631	0·010
	2C	653	91·5	0·1	0·0060	0·0736	0·0394	0·5024	0·010

matter and mucilage which clog the membrane so that filtration rapidly slows down after passage of the first 5 c.c. of the filtrate. As Elford has pointed out, his basis for calculating virus particle size from membrane pore size only holds for the optimum conditions of filtration with minimum adsorption, and such conditions for the filtration of plant viruses have not yet been obtained. It is clear that much remains to be done in studying the influence of various physical factors in the filtration of plant viruses, particularly stability of virus suspensions in relation to medium, *p*H and adsorbability. Some initial attempts have been made at the filtration of a plant virus in a purified suspension and, so far as those attempts go, they do not indicate that a purified suspension has a lower filtration end-point than the same virus in plant sap clarified by passage of a sand and pulp filter or a coarse collodion membrane. This is in agreement with the findings of Elford and Andrewes (1932) on the filtration of purified bacteriophage suspensions.

As regards the purification of plant-virus suspensions, Prof. Keilin suggested to Mr. MacClement and the senior writer that a method used by Warburg and Christian (1932) for purifying a water-soluble ferment might be applied to the purification of a plant-virus suspension, and this method slightly adapted by MacClement (1934) has been used. It may be briefly recapitulated here:

(1) Dilute the crude extracted virus sap at the rate of 1:14, *e.g.* 10 c.c. of virus sap to 140 c.c. of distilled water. Cool to 0° C.

(2) Bubble carbon dioxide through this suspension at 0° C. for half an hour, avoiding as far as possible the formation of ice in the fluid.

(3) Centrifuge for 10 min. (3000 R.P.M.).

(4) Warm 850 c.c. of distilled water to 35° C. Add the supernatant from the centrifuge to this (*i.e.* 1:100 dilution of original fluid).

(5) Pass carbon dioxide through the above for 15 min. at 35° C.

(6) Centrifuge one hour. This period can be reduced somewhat by allowing the fluid to stand overnight in the ice-box.

(7) Discard supernatant. Shake up precipitate which is white and very small in quantity with 10 c.c. of distilled water. This equals the original quantity of virus sap.

(8) Centrifuge till residue to precipitate sticks to the bottom of the tube. Discard residue. The supernatant is the purified virus suspension.

The final result is a colourless, slightly opalescent fluid with a very low protein content. This fluid filters rapidly through even the more impermeable membranes with very little clogging. The above method of purification has so far only been applied by the writers to potato virus *X*, and with this virus the suspension will usually stand a dilution of 1:10,000.

In order to obtain some idea of the reduction in pore size of membranes after passage of virus suspensions a number of discs were recalibrated after use. Discs which had been used for filtering sand and pulp suspensions and those through which purified virus suspensions had been passed were recalibrated and the amount of plugging was compared. It was found that, as a rule, passage of a sand and pulp filtrate reduced the pore size by one-half to one-third, while the amount of reduction in pore size after passage of the purified suspension was very much less. The data are given in Table II.

Table II. *Table showing reduction in pore size of membrane discs after passage of sand and pulp filtrates of undiluted virus sap and purified virus suspensions respectively.*

	A.P.D. of membrane before filtration μ	Virus suspension	*p*H	A.P.D. of membrane after filtration μ
1	0·275	Sand and pulp filtrate	6·2	0·101
1	0·275	CO_2-purified suspension	7·1	0·247
2	0·303	Sand and pulp filtrate	6·2	0·228
2	0·303	CO_2-purified suspension	7·1	0·294
3	0·40	CO_2-purified suspension	7·1	0·362
4	0·136	Sand and pulp filtrate	5·8	0·087
5	0·188	Sand and pulp filtrate	6·6	0·130

Adsorption. It does not lie within the scope of this paper to do more than mention a few facts which have been observed concerning adsorption and retention of the viruses on the membranes.

The concentration of virus suspensions before filtration is of importance, and since this varies greatly according to the virus, age of plant used as source of virus sap and other factors, it has been found necessary to obtain some idea of the titre of virus suspensions by dilution tests prior to filtration. Weak suspensions of virus give a different filtration end-point from suspensions of the same agent with a high virus content.

In comparative filtration with suspensions of the *X* virus in sand-and-pulp filtrates and suspensions purified by the CO_2 method respectively, it was observed that although the purified suspension passed the membrane with

great rapidity the virus was more frequently left behind on the membrane even when the latter was quite permeable. This may be due to aggregation of the virus particles. The following experiment illustrates this. Two suspensions of potato virus *X*, comparable in virus content, were prepared; one was purified by the carbon dioxide method while the other was a Kieselguhr filtrate of undiluted virus sap. Ten c.c. of each of these suspensions were filtered through membranes of the same A.P.D., *i.e.* 240 mμ. After passage of the membrane the filtrate of the Kieselguhr suspension had a high virus content as indicated by inoculation tests to six plants of *Nicotiana glutinosa*, but the filtrate of the purified suspension gave negative results. The surface of the membrane, through which the purified suspension had been filtered, was then lightly scraped into 1 c.c. of water and the scrapings diluted to

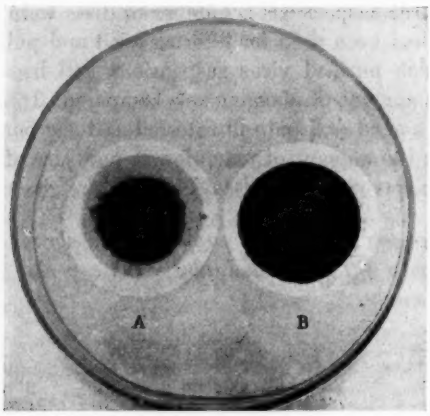

Fig. 7. Two membrane discs of the same A.P.D. through which portions of the same virus sap, but at different *p*H, have been filtered. *A* at *p*H 6·2; *B* at *p*H 8·4. Note the even spread of the filter slime over disc *A* and the concentration of deposit round the holes of the supporting plate in disc *B* indicating a rapid flow at these points.

1:10,000. Inoculations with this suspension gave positive results on two out of three plants. This shows that the virus had practically all remained behind on the surface of the membrane in spite of the fact that the filtration end-point of virus *X* in sand-and-pulp or Kieselguhr filtrates is somewhere round 150 mμ. It may also be mentioned that the 10 c.c. of purified virus suspension passed the membrane so rapidly that practically no pressure was required. Further support for the aggregation theory is given by photographs of the virus in the purified suspension kindly taken by Mr J. E. Barnard with his ultra-violet light microscope. In these preliminary attempts the virus particles appeared to be in aggregations of about 200 mμ in diameter, while the individual particles in the aggregations measured approximately 70 mμ in diameter. If this point be confirmed it would explain why the *X* virus frequently fails to

pass a membrane of 350 mμ A.P.D., while the actual filtration end-point of this virus appears to be near 150 mμ.

The usual *p*H of virus sap from the tobacco plants used in these studies lies between 5·8 and 6·6 as determined colorimetrically. It was found that the rate of filtration differed markedly according to the *p*H. Virus suspensions at a *p*H of 8·4 filtered at less than half the pressure and in a fraction of the time necessary for filtration of the same virus suspension at *p*H 5·8–6·2. In Fig. 7 are shown two membrane discs of the same A.P.D. through which portions of the same virus suspension but at different *p*H have been filtered. *A* is the disc through which the virus sap at *p*H 6·2 has been passed, while similar virus sap at *p*H 8·4 has been filtered through *B*. It will be noticed that the filter slime is spread evenly over the surface of the disc *A* without any concentration of deposit around the holes of the supporting plate underlying it, while in *B* there is great concentration over the perforations in the plate indicating a rapid filtration flow at those points.

Although the *rate* of filtration varies according to the *p*H there is as yet no evidence that changing the *p*H of a virus suspension enables the virus particles to pass a membrane of smaller A.P.D. than they would otherwise have done.

Summary

An account is given of the preparation of Elford's gradocol membranes and their application in the study of plant viruses. The technique is discussed step by step and the chief sources of error are indicated. It is shown that plant virus suspensions clog the membranes, and suggestions are made for the preparation of those suspensions for filtration. The effect of virus content, purification and *p*H of virus fluids upon their filtration is discussed.

Acknowledgments

Grateful acknowledgment is due to Prof. D. Keilin, F.R.S., who not only gave the writers all facilities for this work at the Molteno Institute, Cambridge, but followed its progress throughout with advice and encouragement, and to Dr W. J. Elford for much assistance and advice always most willingly given. Acknowledgment is also due to Dr A. A. Miles and Dr E. T. C. Spooner for their kind help in supplying the various bacteriophages and cultures of *B. prodigiosus*, to members of the staff of the Biochemical Institute for kindly preparing the special reagents, to Mr W. D. MacClement for some suggestions made during the course of the work, and to Mr Charles Harpley for assistance in matters of technical detail.

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NOTES ON THE DISTRIBUTION OF THE LARVAE AND PUPAE OF *SIMULIUM* SPP. IN THE RIVER TEES AND ITS TRIBUTARIES

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(With 1 Figure in the Text)

The study of the fauna of rivers is greatly handicapped by the virtual impossibility of identifying many aquatic larvae of insects. In regard to the river Tees, where the fauna consists mainly of the larvae of Diptera, Trichoptera, Ephemeroptera and Plecoptera, this limitation was of considerable importance in the ecological survey of the fauna of the upper river carried out as part of the programme of the Water Pollution Research Board of the Department of Scientific and Industrial Research.

The immature stages of the British species of the genus *Simulium* (Diptera Nematocera) have, however, been satisfactorily described by Edwards (1920–1) and Puri (1925), and it is practicable to refer larvae and pupae of this genus to their species. The distribution of the various species in the Tees and its main tributaries presents some features which may be worth describing in view of the blood-sucking habits of certain species.

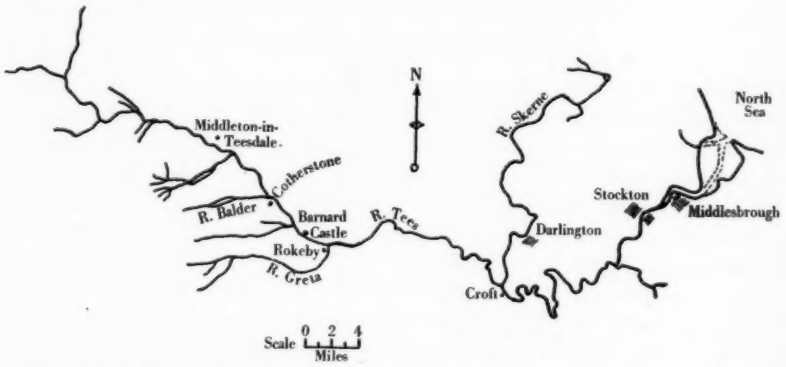
It may be pointed out that while the identification of *Simulium* pupae is easy and certain, the determination of larvae is less certain, as it is based largely on the structure of the gills which are frequently retracted, and on the colour pattern of the head, which in some species is very faint. Although some larvae are quite unmistakable, as a rule the presence of any species has been regarded as established only when pupae as well as larvae have been found.

Topography

The Tees (see map) rises on the slopes of Cross Fell at a height of 2500 ft. above sea-level; it flows eastward and has a total length of 100 miles, being tidal for the last 25 miles of its course. For the first 50 miles from the source the river bed consists mainly of millstone grit and mountain limestone, with some basalt in the High Force region and magnesian limestone in the 10 miles above Croft. From Croft to the sea the river flows over a thick bed of glacial drift and its character changes completely. Above Croft it is a typical mountain stream of alternating pool and rapid, with mainly rocky banks, while below it is a winding lowland river, with soft banks, although in many places the current is rapid. The tributaries examined were the Balder, which rises on Stainmoor and flows over millstone grit to join the Tees at Cotherstone, the Greta, also rising on Stainmoor, with a bed of mountain limestone and millstone grit joining the Tees at Rokeby, and the Skerne, a stream of a different type which

rises on a low ridge near Trimdon, and flows through flat country over a bed of glacial drift overlying magnesian limestone, to join the Tees at Croft.

The water of the Tees, Greta, and Balder is rather soft and slightly alkaline; it is frequently coloured brown by peaty materials from the moors which these rivers drain. The Tees water below Croft is harder than it is in the upper reaches, largely owing to the influence of Skerne water which is very hard and never coloured by peat.



The following analyses, for which the writer is indebted to his former colleague Dr J. Longwell, are typical of the various types of water found in this area.

Table I

	Upper Tees	River Balder	River Greta	Lower Tees	River Skerne
Temperature ° C.	8·2	12·1	11·4	11·0	16·8
Dissolved oxygen, parts per 100,000	1·08	1·1	1·0	1·06	0·9
% saturation	89·7	99·5	90·5	97·7	90·9
Free and saline ammonia, parts N per 100,000	0·005	0·005	0·005	0·03	0·02
Temporary hardness, parts $CaCO_3$ per 100,000	2·5	3·5	8·0	10·0	26·1
Chloride, parts Cl per 100,000	0·85	1·15	1·15	1·65	8·05
*p*H	7·5	8·1	8·0	7·9	8·0

In the Balder, Greta and Tees above Croft vegetation is restricted to mosses and algae; below Croft flowering plants occur in some quantity, while in the Skerne they are abundant.

The distribution of *Simulium*

Simulium larvae are very abundant in the Tees and its tributaries and are found, frequently in enormous numbers, wherever the current is rapid. In the upper reaches of the Tees and in its moorland tributaries they live on rocks and stones, while in the lower reaches and the Skerne they are found mainly, but not exclusively, on the water plants.

In the upper Tees, the Balder and the Greta the dominant species are *S. monticola* Friedrichs and *S. variegatum* Meigen, and indeed, in the Balder these two species only have been recorded. In the Tees and Greta *S. reptans* Linn. *sensu stricto* occurs regularly associated with the other two species in all the fast-flowing reaches examined. All the pupae and, with three exceptions, all the larvae, found in the Tees and its tributaries above the entry of the Skerne, were referable to these three species. The exceptions were firstly the discovery on two occasions of a solitary larva of *S. equinum* Linn. in comparatively slow reaches, where *Simulium* larvae of any species seldom occurred, and secondly on one occasion of some larvae which appeared to belong to *S. tuberosum* Lundstrom were found. The identification of these species was not confirmed by finding the pupae, and while the larvae of *S. equinum* are distinctive and there can be no doubt of their identity, those of *S. tuberosum* closely resemble those of *S. reptans*, and in the absence of confirmatory pupae the record must be regarded as doubtful.

Below Croft, where the Skerne enters the Tees and where the river bed is composed of glacial drift, the dominant species are *S. equinum* Linn. and *S. reptans* var. *galeratum* Edwards accompanied regularly by *S. reptans* Linn. *sensu stricto* in smaller numbers. It may here be noted that although the larvae of *S. reptans* Linn. *sensu stricto* and *S. reptans* var. *galeratum* are easily distinguishable, the pupae are apparently exactly similar. The pupae found in the lower reaches of the Tees consisted exclusively of those of *S. equinum* and *S. reptans*. The larvae of these two species made up practically the whole of the *Simulium* fauna, but larvae apparently belonging to *S. variegatum*, *S. monticola*, *S. tuberosum*, and *S. aureum* Fries, were found on one occasion.

The predominant species in the Skerne is *S. ornatum* Meigen. It occurred in all the fast stretches examined; in two places it was accompanied by smaller numbers of *S. aureum* and, at the highest point of the river examined, pupae of *S. reptans* were also present, but elsewhere it occurred alone.

In the Tees area, therefore, there are three distinct regions each populated by its own *Simulium* fauna. These may be summarised as:

(1) The upper Tees, where the predominant species are *S. monticola* and *S. variegatum*.

(2) The lower Tees, where the predominant species are *S. equinum* and *S. reptans* var. *galeratum*.

(3) The Skerne, where the predominant species is *S. ornatum*.

DISCUSSION

There are very few data with which the foregoing observations can be compared, and it is obvious that no explanation of them can yet be given. In general the habitats for the various species are similar to those recorded by Edwards (1920–1); *S. monticola* and *S. variegatum* are found in the mountain streams and *S. ornatum* and *S. equinum* are associated with water plants. *S. reptans* var. *galeratum* has been recorded only twice, once in the River Otter,

Devon, where it occurred, as it does in the Tees, on weeds and stones (Edwards, 1920–1), and once from Tännforsen Falls, Sweden, and obviously more data are needed before its habitat can be defined, but it also appears to be associated with water plants. It seems reasonable to correlate the absence of *S. ornatum* and *S. equinum* and probably also *S. reptans* var. *galeratum* from the upper river with the absence of macrophytic vegetation, and it seems likely that some change in the river at Croft makes the lower reaches unsuitable for "mountain" species. The reason why the water plants of the Skerne should shelter *S. ornatum* while the same species of plant in the Tees shelter *S. equinum* is at present quite unknown. The fauna of the Skerne is, in many respects, markedly different from that of the Tees. A full account of these differences will shortly be published in the Report of the Survey of the River Tees as a Technical Paper of the Water Pollution Research Board of the Department of Scientific and Industrial Research, and it is sufficient here to say that while the fauna of the Skerne consists largely of Crustacea and Mollusca, that of the Tees consists principally of insect larvae. These differences seem to be connected in some way with the calcium content of the waters (Thienemann, 1912), those with a high calcium content having a fauna of the Crustacea-Mollusca type. It may be that in hard waters *S. ornatum* occupies the ecological niche which in softer water is inhabited by *S. equinum*, but knowledge of river biology is too fragmentary to offer any satisfactory explanation of these curiosities of distribution.

SUMMARY

1. The distribution of the larvae of *Simulium* spp. in the River Tees area is discussed.

2. The dominant species of
   (*a*) the upper Tees are *S. monticola* and *S. variegatum*;
   (*b*) the lower Tees are *S. equinum* and *S. reptans* var. *galeratum*;
   (*c*) the Skerne is *S. ornatum*.

3. The causes of these differences are unknown, but it is suggested that geological structure, the distribution of water plants and the hardness of the water are important factors.

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THE EFFECT OF *PROTEOSOMA* UPON THE SURVIVAL OF *CULEX*

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I. Problem

Many workers must have asked themselves whether a malaria parasite of bird or mammal has any harmful effect on the mosquito in whose body it grows and multiplies so remarkably; but though various opinions have been expressed, I cannot discover that any conclusive answer to this question has been given.[1]

II. Methods

The mosquitoes used in these experiments were *Culex fatigans*, bred in London for many generations but derived originally from India. The larvae were fed on an infusion made by pouring boiling water over hay and allowing it to stand for 48 hours; this infusion was strained and used as food for 1 day only. The larvae were removed with muslin every day and transferred to a new infusion made in the same way and 48 hours old. We believe that an ample supply of food was always available, and the growth of the larvae was very regular.

I made preliminary studies on the length of life of mosquitoes fed on uninfected birds and kept under standardised conditions. The newly emerged adult females were kept for 3 days in a moist room at about 30° C. and a bird was then put in the cage. Next morning those females which had taken a full meal of blood were isolated and starved. In one set of observations they were put at 23° C. and 60 per cent. relative humidity. The females were put singly in small lengths of glass tubing closed at each end with gauze and stored in fruit jars over sulphuric acid adjusted to give the required relative humidity (Buxton and Mellanby, 1934). The insects were given no further food or drink, and deaths were recorded daily. Under these experimental conditions death occurred as follows:

Day ...	1	2	3	4	5	6	Total
Deaths	4	27	66	89	42	18	246

The figures show that there is a high degree of inconsistency between one female and another in spite of the carefully standardised technique. We conclude that if infection with *Proteosoma* shortens the life of these insects, it will

[1] I observe that Duke (1928) has published a large body of fact, accumulated in the course of other studies, on the length of life of bred *Glossina palpalis*, some of which were infected with *Trypanosoma gambiae* or *rhodesiense*. He reaches the conclusion that the infection is not injurious to that insect.

only be one factor among many. It may, therefore, be difficult to obtain a convincing proof of its existence.

III. Experiments

The experiments were carried out with the strain of *Proteosoma* isolated in Rome by Raffaele, which was put at my disposal by Sir Rickard Christophers. The organism is apparently *Proteosoma praecox*, though the identification is not very critical. The *Culex fatigans* were fed on a canary, the blood of which contained ripe gametocytes. It is known from dissecting those which died that over 90 per cent. actually developed oocysts. Control mosquitoes were fed on a clean bird, and both groups of insects were isolated precisely in the manner already described except that they were kept at 30° C. and 90 per cent. humidity. Experiments of this nature were repeated until a considerable body of fact had been accumulated, and the deaths among the controls and infected mosquitoes were found to be as follows:

Day ...	1	2	3	4	5	Total
Control	1	19	97	22	2	141
Infected	11	71	143	27	3	255
Totals	12	90	240	49	5	

It will be seen at a glance that on the first and second days the proportion of deaths is greater among the infected than among the control mosquitoes; but only a very bold or ignorant person would draw any conclusion from the figures as they stand, for we know how inconsistent are the results even when the insects are bred and treated in an identical manner. We may, however, test the significance of the difference between the control and infected mosquitoes by making a hypothesis that infection with *Proteosoma* has no effect at all. We can then take the "observed" deaths and calculate what would be "expected". There were altogether 141 control and 255 infected mosquitoes: if infection had made no difference, we should expect the deaths on each day to be divided in this proportion between control and infected groups. For instance, on the first day 12 deaths occurred, 1 control and 11 infected; but on the hypothesis we should have expected 4·3 and 7·7 (obtained by dividing 12 in the proportion 141 : 255). In this way we obtain the following expected figures, the total number of deaths on each day being the same as the totals in the observed figures.

Day ...	1	2	3	4	5	Total
Control	4·3	32	85·4	17·4	1·8	141
Infected	7·7	58	154·6	31·6	3·2	255
Totals	12	90	240	49	5	

If the hypothesis (that infection does not contribute to deaths) is consistent with the facts, the difference between observed and expected figures will be slight; if the difference is great, we may take it as evidence that the hypothesis is erroneous. The difference may appropriately be measured by χ^2 (Fisher,

1934, chap. 4); in the present instance the value of χ^2 is 16·5 with 4 degrees of freedom. So great a value of χ^2 would occur less than 1 in a 100 times, which is so unlikely that we may regard the hypothesis as disproved. In other words, I conclude that *Proteosoma* had a significant effect on the daily deaths. As, moreover, the largest contribution to χ^2 was on the first and second days, it is confirmed that those are the days on which a significant increase of deaths occurred.

Experiments, similar in all other respects, were carried out at 30° C. and 60 per cent. humidity with the following results:

Day ...	1	2	3	4	Total
Control	5	87	19	1	112
Infected	15	63	8	0	86

Here again is a conspicuous increase in deaths on the first day among the infected mosquitoes. One cannot assess the value of the figures as they stand, but testing by χ^2 it is found that the probability is between 1 : 20 and 1 : 50. So that here again is a significant difference between the groups, and the difference occurs on the first day.

It would be fair comment to say that though the point has been proved, the conditions are unnatural, for the mosquitoes are starved to death after a single blood meal. What result would one obtain if they were kept alive longer, perhaps by some vegetable food given daily after the blood meal? I have made a group of experiments to study this point. The control and the infected mosquitoes came from the same population, and were fed on different birds on the same night; we know positively that infection occurred, for when it was possible the dead mosquitoes of the infected group were dissected, and out of 21 stomachs oocysts were found in 17. In these experiments the mosquitoes were kept at 90 per cent. humidity and 23° C., and they were given slices of fresh apple for 3 hours daily, for it was thought undesirable to leave the apple with them permanently owing to its disturbing effect on the humidity equilibrium. The results are given in Table I, in which the three separate experiments and the totals will be found. An increase in deaths on the second and third days in the infected group can be observed consistently in each experiment. It appears also that there is a second increase about the seventh day. Taking the total figures and testing them by χ^2, we find that the probability is less than 1 : 100. Here again, then, we have conclusive proof that infection caused an increased mortality; and one can see from the daily contributions to χ^2 that the increase in deaths among the infected mosquitoes was on days 2–3 and 7. There was a noticeable decrease on day 5.

After the experiments with apple were concluded, we discovered that *Culex fatigans*, after a single blood meal, lives very much longer if fed with raisins than with apple. I attempted to make use of this, hoping to discover whether the infected mosquitoes showed an increased mortality still later in life (say after the seventh day, at 23° C.). Before the experiments were concluded, some unknown factor began to cause all the mosquitoes to die unusually early

As a conclusive result had been reached with the other experiments, it seemed unnecessary to start the work again.

Table I. *Showing deaths daily, in control mosquitoes fed on canary, and in "infected" mosquitoes fed on a canary whose blood contained ripe gametocytes. After the blood meal, the insects were maintained at* 23° C. *and* 90 *per cent. relative humidity, and given apple* 3 *hours daily.*

Day ...	1	2	3	4	5	6	7	8	9	10	Later	Total
Control	0	0	1	5	1	0	1	0	0	1	2	11
Infected	0	2	2	4	0	1	1	0	2	0	1	13
Control	0	0	0	7	5	1	1	0	2	1	4	21
Infected	0	0	3	9	0	1	2	0	0	0	3	18
Control	0	0	0	1	7	6	2	0	0	1	1	18
Infected	0	0	3	1	3	5	6	0	0	0	0	18
Total:												
Control	0	0	1	13	13	7	4	0	2	3	7	50
Infected	0	2	8	14	3	7	9	0	2	0	4	49
% living:												
Control	100	100	98	72	46	32	24	24	20	14		
Infected	100	96	80	51	45	31	12	12	8	8		

IV. Discussion

These experiments prove that if females of *Culex fatigans* are infected with *Proteosoma*, deaths occur earlier than in controls: at 30° C. the effect of infection is apparent on the first day, at 23° C. on the second. This is doubtless due to invasion of the wall of the midgut by the ookinete. In the last set of experiments at 23° C. (Table I), I have also demonstrated an increased number of deaths on the seventh day among the infected mosquitoes. This apparently occurs before the rupture of the sporocysts, but it may be due to their growth.

It will be observed that the experiments were performed under different conditions of nutrition and of climate, and a significant effect of parasitisation observed on each occasion. It may therefore be supposed that a similar effect of infection with *Proteosoma* occurs in nature. It does not necessarily follow that infection of *Anopheles* with *Plasmodium* would tend to shorten the insect's life, but the matter is open to experimental study.

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OCCURRENCE OF A LARVAL CESTODE IN THE RED LOCUST *NOMADACRIS SEPTEMFASCIATA* SERV.

By A. M. GWYNN and A. G. HAMILTON

From the Imperial Institute of Entomology, London

(With 4 Figures in the Text)

Introduction

A parasite which proves to be the cysticercoid of a Cestode was discovered by one of us (A. M. G.) in the course of work on the locust problem in British Chad in 1933–4. The material was sent to the Imperial Institute of Entomology, London, where the other author (A. G. H.) undertook the microscopic examination, measurement and description of the specimens and determined their nature. This is the first time a Cestode larva has been recorded in Acrididae and, as far as can be ascertained, in Orthoptera generally. The original slides have been deposited at the British Museum (Natural History).

Occurrence

A number of adult specimens of *Nomadacris septemfasciata* Serv., bred from hoppers in September 1933, were kept in a cage in the grass hut which served as a laboratory during the work. The numbers were gradually reduced by losses due to death and escapes till there were only six left, which were all killed on January 15th, 1934, after having been in captivity for nearly 4 months. Among the specimens killed was a small female with deformed elytra which had attracted attention earlier owing to its peculiar pallid colouring. During their captivity the other locusts in the cage had undergone remarkable changes in colouring till they were liver-coloured practically all over, with brick red markings on the head, elytra, etc.; this was completely different from the slight colour changes which took place in specimens in the field, and must have been due to something in the unnatural conditions under which they were being kept. The pallid female, however, hardly changed in colour, except to become even paler. An interesting difference in behaviour was noticed; while the other locusts spent nearly all their time on the dark wire gauze of the cage clustered at the corner nearest to the light, the pallid female was always to be seen on the grey-green grass in the cage, to which colour it approximated. When the six survivors were killed, the pallid female and one other were dissected, and in the former were discovered the parasites which are the subject of this paper.

At the end of January 1934, 100 specimens of *Nomadacris* collected in the field were dissected, primarily to look for dipterous parasites. In June and July 1934 numerous females of both *Nomadacris* and of *Locusta migratoria migratorioides* R. and F., were dissected for the state of the ovaries to be examined, and on one occasion twenty-two male *Nomadacris* were dissected for parasites. Cysticercoids were never found again, so that this remains the only record of their occurrence. The locust in which the cysticercoids were found was the only pallid specimen dissected, but during October–December 1933 very occasional pallid specimens of adult *Nomadacris* similar to the one described were seen in the field. At the time it was thought that they might represent an albino race of *Nomadacris*, such as Faure (1932, p. 398) describes in the case of *Locustana pardalina* Walk., but in the light of the observations of Michelmore and Allan (1934), referred to below, it is probable that they were parasitised either by cysticercoids or by some other parasite.

One of us (A. M. G.) returned to the Chad area in May 1934 after an absence of 3½ months, and remained there till the following November. During this period a watch was kept for further pallid specimens, but none were seen either among the survivors of the 1933 generation which died out during June and July 1934, or among the 1934 generation which became adult towards the end of September. The numbers of *Nomadacris*, particularly in the case of the 1934 generation, were very much smaller during this period than they had been at the end of 1933.

During the work in this area considerable attention was paid to the other species of Acrididae present, but in none of them were pallid specimens observed.

General description

When the locust was dissected the parasites were observed in the abdominal cavity as whitish ovoid bodies situated at the end of Malpighian tubules. On examination it was found that these bodies were cysts, each containing what appeared to be a complete cysticercoid. Throughout the rest of this paper these are referred to as the cyst and cysticercoid, because we were unable to decide whether the outer cyst was due to proliferation of the tissue of the host or whether, as Dr H. A. Baylis has suggested, it was the degenerated blastogene as figured by Mrazek (1907, Text-fig. F, and Pl. 31, fig. 13). It appeared to be composed of large multinucleate cells, but the material was not very well preserved for studying histological details. The shape of the cysts varied greatly as shown in Figs. 1–3; many were dumb-bell-shaped, as in Fig. 3. The cysticercoid was situated towards the distal end of the cyst, with the scolex pointing in that direction (Fig. 4). The measurements shown below were made on unmounted specimens, cleared to show the position of the cysticercoid.

In searching for these bodies care should be taken not to confuse them with the salivary glands which occur in the thorax and which are about the same size, though otherwise there is little resemblance between the two.

THE CYSTICERCOID

Systematically all that can be said definitely of the parasite is that it is a cysticercoid with an unarmed scolex. By elimination of the families to which the cysticercoid is unlikely to belong one is left with the probability that it belongs to either the Dilepididae, the Hymenolepididae or the Anoplocephalidae. The early stages of the Anoplocephalidae are still unknown, but

Table showing measurements (in mm.) of cysts and cysticercoids.

	Length			Width		
	Average	Min.	Max.	Average	Min.	Max.
Cyst	1·59	0·94	2·80	0·79	0·66	0·96
Cysticercoid	0·63	0·60	0·66	0·30	—	—

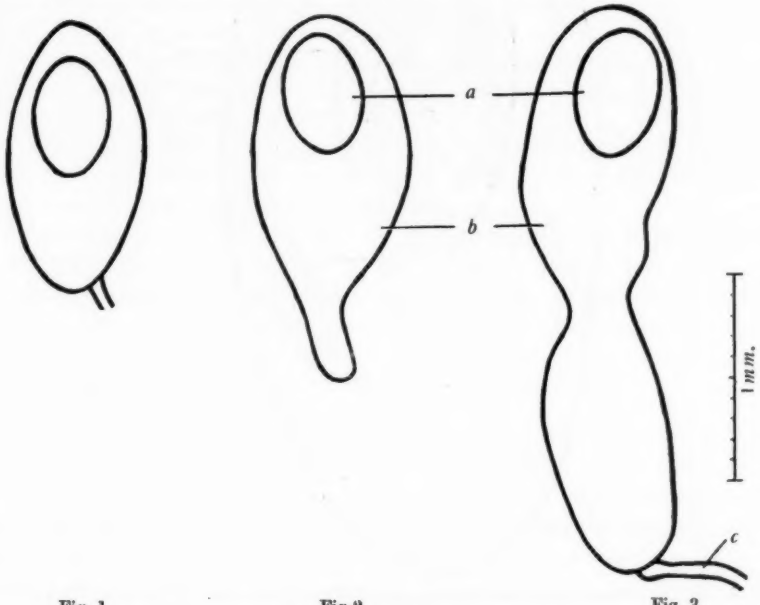

Fig. 1. Fig 2. Fig. 3.

Figs. 1–3. Cysts of various shapes. *a*, cysticercoid; *b*, cyst; *c*, Malpighian tubule.

the family cannot be ruled out on that account. The apertures of the suckers in this cysticercoid appear to be overhung by lappets originating from their anterior margins. These lappets are indicated somewhat diagrammatically in Fig. 4, but in some of the sections they appeared to be quite well-developed structures. Dr Baylis has pointed out the resemblance of the cysticercoid in this respect to the genus *Octopetalum* Baylis 1914 (Dilepididae, subfamily Paruterininae), the two known species of which are parasites of guinea-fowl.

Possible life cycle

This is probably the cysticercoid stage of a Cestode occurring in some bird or mammal which preys on the locust, the eggs being ingested by the locust on contaminated blades of grass. From the gut of the locust the eggs must apparently pass up the Malpighian tubules, at the end of which they develop into cysticercoids. For their cycle to be completed the locust must presumably

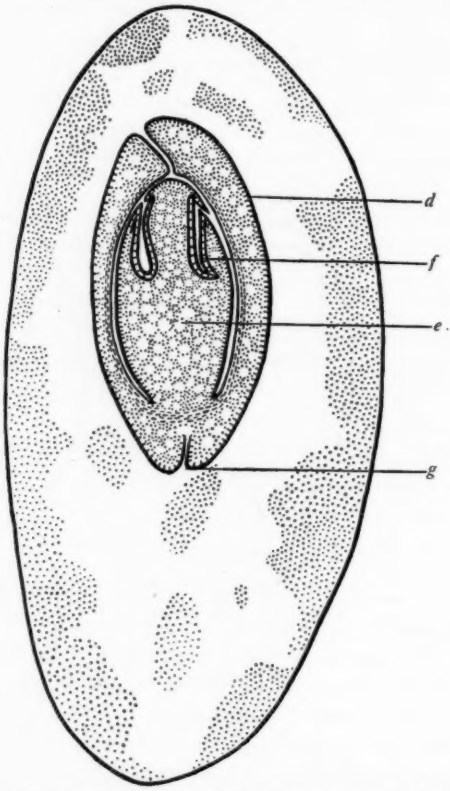

Fig. 4. Slightly reconstructed longitudinal section of complete cyst. *d*, cysticercoid; *e*, scolex, *f*, sucker; *g*, invagination at posterior end (cf. Mrazek's fig. 13).

be eaten by the final host. In the Chad region the most conspicuous predators of these locusts were the Carmine Bee-Eater (*Merops nubicus* Gmelin), the Cattle Egret (*Bubulcus ibis* L.) (Golding, 1934, p. 298) and the Great Egret (*Casmerodius albus melanorhynchus* Wagler), though in the case of the egrets the likelihood that they prey on this locust is only inferred from their behaviour. Many other birds must also prey on these locusts occasionally, and the latter may form an important item in the diet of some of the bustards which occur in this area.

OCCURRENCE IN OTHER INSECTS

Though not previously recorded from Orthoptera, several Cestodes are known to occur normally in insects of other orders in their cysticercoid stage (Joyeux, 1920; Hall, 1929; and others). In the literature consulted they are described as occurring free in the body cavity of the insect concerned; the cysticercoids under consideration appear to be unique in being attached to the end of the Malpighian tubules of their host, a fact which may be connected with the large size of their host as compared with the insect hosts recorded by other authors.

Michelmore and Allan (1934, p. 115) describe a pallidness of hoppers in *Nomadacris*, due to parasitism by Nematodes, which, however, killed the hoppers before they became adult in the cases observed. Dipterous larvae were also occasionally found in pallid hoppers, but it seems doubtful whether they were responsible for the pallid condition. These authors also record finding pallid hoppers apparently unparasitised, and suggest that they may have been hoppers from which the Nematode had emerged, though in captivity hoppers only survived the emergence of this parasite "for a day or two". We venture to suggest that these apparently unparasitised pallid hoppers may have contained cysticercoids such as those described in this paper; these would probably have been overlooked, as Michelmore and Allan state that the pallid hoppers were "pulled open", presumably in the field.

As to the reason why infestation by certain parasites should produce this pallid form, the fact that the cysticercoids infect the Malpighian tubules may be of importance. It is possible that pallidness in the host results only when the parasites are exceptionally numerous, and that they may occur in smaller numbers comparatively frequently.

ACKNOWLEDGMENTS

We are indebted to Mr H. R. Hewer, of the Royal College of Science, London, for assistance in identification and description of the material; to Dr H. A. Baylis of the Natural History Museum, who also examined the material and gave great assistance on points of systematic importance and literature; and to Mr B. P. Uvarov for his advice and criticism.

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THE LARVA OF *PHAONIA MIRABILIS* RINGDAHL, PREDATORY ON MOSQUITO LARVAE (DIPTERA, ANTHOMYIDAE)

By P. TATE

From The Molteno Institute, University of Cambridge

(With 8 Figures in the Text)

Keilin (1917) described the mature larva and puparium of an Anthomyid, the imago of which was described by Collin as *Phaonia keilini* n.sp. Ringdahl (1923) pointed out that this species had been described by him as *P. mirabilis* previously, and consequently *P. keilini* fell into synonymy with *P. mirabilis* Ringdahl 1916. Keilin's material consisted of only a few mature larvae collected from decaying detritus at the bottoms of holes in elm trees, and his description was limited to the general anatomy of the mature larva, based chiefly on the third moults extracted from puparia, and of the puparium. Keilin considered that the larval structure, especially as regards the mouthparts, indicated that the larvae are carnivorous.

In May 1931 five eggs of *P. mirabilis* were found floating on water in a reservoir in a horse-chestnut tree trunk standing in the garden of the Molteno Institute, Cambridge (this reservoir had previously yielded a very interesting fauna described by Keilin (1927)). In July 1933 four more eggs and an empty egg shell were found floating in a very small reservoir in an ash tree near Grantchester, Cambridge. The eggs are white, 1·8 mm. long and 0·7 mm. broad, ventrally keeled, have lateral flanges and are marked with delicate hexagonal sculpturing on the dorsal surface (Fig. 7). Larvae and one female imago were bred from these eggs. The eggs hatched 3–4 days after they were collected. The very small larvae which emerged were remarkable in having all the characters of the typical third instar Cyclorrhaphous larva. The fact that these characters are present in larvae dissected out of the egg shows that the presence of these characters is not due to rapid moulting after the larva hatches. If a larva is examined immediately after it has hatched, or has been dissected out of the egg, it is seen that four or five lobed anterior spiracles are present, the posterior spiracles are triforous and the bucco-pharyngeal armature consists of paired lateral hooks (Fig. 1, *l.h.*), median ventral arc (*m.v.a.*), oral bars provided with long curved teeth (*o.b.*), and a posterior ribbon (*p.r.*). The structure of the bucco-pharyngeal armature is similar to that described by Keilin in the mature larva except that the elements, notably the posterior ribbon and the median ventral arc, are less

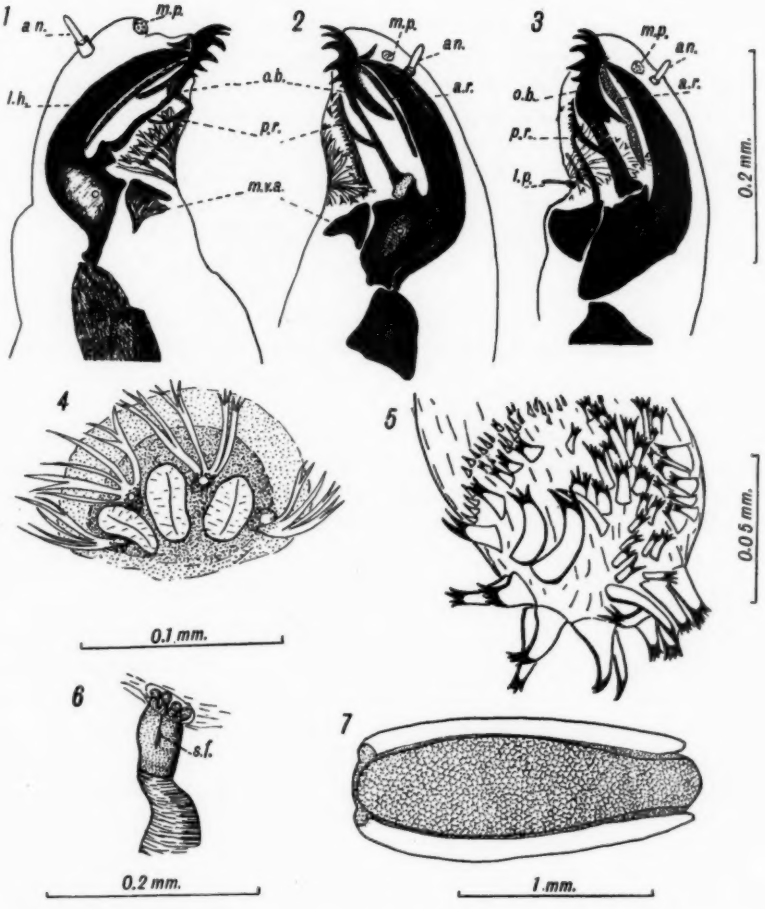

Fig. 1. Lateral view of head of larva dissected out of egg.
Fig. 2. Head of a larva 1 day old.
Fig. 3. Head of moult extracted from puparium.
Fig. 4. Posterior spiracle of larva extracted from the egg.
Fig. 5. One of the anterior pair of abdominal protuberances, showing multi-pointed hooks.
Fig. 6. Anterior spiracle of a larva 1 day old.
Fig. 7. Egg, dorsal aspect.

an. antenna; *a.r.* anterior ribbon; *l.h.* lateral hooks; *l.p.* labial palp; *m.p.* maxillary palp; *m.v.a.* median ventral arc; *o.b.* oral bars; *p.r.* posterior ribbon; *s.f.* scar filament.

strongly chitinised and the anterior ribbon (Figs. 2, 3, *a.r.*) is not easily distinguishable. Figs. 1, 2 and 3 are drawn from a larva extracted from the egg, a larva 1 day old, and from a moult withdrawn from the puparium, respectively, and it is clear that, except for increased chitinisation with age, the size and arrangement of the elements are the same in each case. Deep oral grooves radiate from the mouth on each side and there is a dermal tooth between the distal ends of each pair of grooves. In addition to antennae (*an.*) and maxillary palps (*m.p.*) described by Keilin (1917), labial palps (Fig. 3, *l.p.*) are visible in the mature larva. The oral bars (*o.b.*) terminate in a group of three large recurved teeth posterior to which there is a group of three smaller teeth. Owing to the heavy chitinisation only two of the latter group are obvious in the mature larva, but careful examination reveals the third posterior tooth.

The three paired groups of three long, slender, mobile hairs described by Keilin on the ventral surface of the thoracic segments are present and equally well-developed in the young larvae. These hairs appear to be sensory in function and represent the vestiges of the legs.

Paired retractile protuberances are present ventrally on the second to eighth abdominal segments. They bear large sharply pointed curved hooks directed posteriorly on the first seven pairs and anteriorly on the last pair situated on the eighth abdominal segment. A point not previously recorded is that the large hooks on the first pair of protuberances have not simple points but end in grapnel-like crowns of very fine spines, the number of which may vary from two to six or even more (Fig. 5).

Although not sufficient material was available for a detailed study of the respiratory system there are several interesting points concerning it. Thus, although apparently no moults take place during larval life, in larvae only 24 hours old faint but definite external scars and scar filaments are discernable in both the anterior (Fig. 6, *s.f.*) and posterior spiracles. The anterior spiracles usually have five papillae but sometimes only four (Fig. 6). The openings of the triforous posterior spiracles are covered with a thin membrane provided with a medial slit (Fig. 4). The main lateral tracheal trunks expand into two large reservoirs, one lying in the second and third thoracic and first abdominal segments (Fig. 8, *T.B.*) and the other extending from about the third to fifth abdominal segments (Fig. 8, *A.B.*). Near each posterior spiracle a short, stout, trachea is given off which ends in a swollen ampulla (Fig. 8, *P.B.*).

Biology

The larva hatches by forcing off a ventrally hinged cap at the anterior end of the egg. The larvae were reared either in water taken from the tree reservoir or in tap water containing some pieces of partly submerged rotten wood. The larvae are very active, crawling rapidly over the surface of the wood, swimming freely suspended by the posterior spiracles from the surface film, or moving actively completely submerged. Frequently they attach themselves to pieces of wood by gripping it between the last and penultimate pairs

of ventral protuberances. When so attached the anterior part of the body is extended and sways about in the water. When suitable prey is touched the larva rapidly encircles it with the anterior part of its body, gripping it firmly with the extended ventral protuberances, and perforating it with the mouth-parts. Mosquito larvae are readily attacked and the specimens of *Phaonia mirabilis* reared were fed on larvae of either *Aëdes geniculatus* or of *Culex pipiens*. Within a few hours of hatching the *Phaonia* larvae can capture prey. Once the prey is punctured its contents are scraped out by the mouth-parts of the larva, and from time to time, as the contents are consumed, the *Phaonia* larva shifts its grip so as to bring fresh tissue within reach of its mouth-parts, until only the empty skin of the prey remains. The larvae are very voracious and it is noticeable that many more prey are killed than are eaten, and also that for a few days before pupation they continue to kill prey but do not feed. Records were kept in the case of one *P. mirabilis* larva, and during the last two weeks of larval life it destroyed eighty large mosquito larvae. Since, in

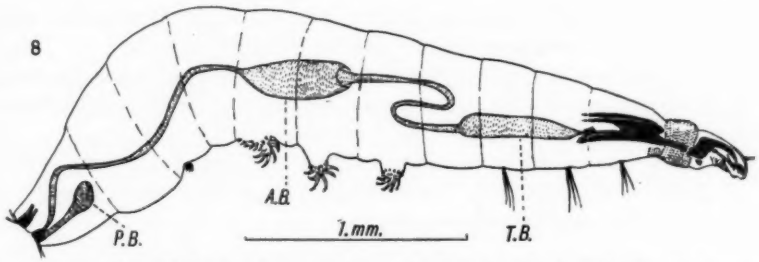

Fig. 8. Larva 1 day old showing the expansions of the lateral tracheal trunk. The specimen was dead and much distorted, the anterior part is seen in lateral view and the posterior in dorsal view.

A.B. abdominal expansions; *P.B.* posterior expansions; *T.B.* thoracic bladder-like expansions.

the specimens reared, the duration of larval life of *P. mirabilis* was about one month, the total number of mosquito larvae which one *Phaonia* larva can destroy must be more than one hundred.

Pupation took place between pieces of rotten wood a short distance above the surface of the water, and the one female which was obtained emerged about two weeks after pupation. The puparium, as was described by Keilin, is thin and semi-transparent so that the pupa within can be easily observed.

DISCUSSION

The most interesting point in the life history of this Anthomyid is the absence of the customary trimorphism during larval life. This phenomenon has been described previously in only three cases. Portchinsky (1910) described it in the case of *Mydaea urbana* Mg. and *Myospila meditabunda* F., and Keilin (1917) described the third case in *Melanochelia riparia* Fall. This

precocious development of the third instar characteristics no doubt is correlated with the carnivorous mode of life and the necessity for seizing and devouring prey from the beginning of larval life.

The presence of the bladder-like expansions of the main tracheae can also be correlated with the mode of life. These expansions probably serve as reservoirs for air when the larvae move about completely submerged. The retention of external scars and scar filaments in the spiracles is an interesting case of the persistence of vestigial structures in view of the apparent absence of moults during larval life.

The grapnel-like spines on the first pair of ventral protuberances are important in the prehension of the active prey. It is only when the prey is firmly gripped by the anterior body segments that the *Phaonia* releases its anchorage by the posterior end and further encircles the prey.

Summary

1. *Phaonia mirabilis* eggs are laid on the surface of water in tree holes. The eggs are white and have lateral flanges and a ventral keel.

2. Trimorphism is absent during larval life, and, except for size, the newly hatched larva resembles the mature larva, having anterior spiracles, triforous posterior spiracles and complete buccal armature.

3. The larvae are carnivorous and capture and devour mosquito larvae.

4. Eggs hatch in 3–4 days. The duration of larval life is about 4 weeks and that of the pupal stage about 2 weeks.

Acknowledgment. I wish to express my thanks to Prof. D. Keilin for advice during the course of the work and to Mr J. E. Collin of Newmarket for identifying the imago.

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THE VALUE OF THE EXTERNAL SPINE OF THE HARPAGO IN IDENTIFYING VARIETIES OF *ANOPHELES MACULIPENNIS*

By P. G. SHUTE

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(With Plate XIX)

In "A Brief Guide to the Varieties of *Anopheles maculipennis*" which was published in the *Quarterly Bulletin of the Health Organisation of the League of Nations*, 3, No. 4, December 1934, it is recorded that five varieties of this species are considered valid and that there is in addition the closely allied species—or a further variety—*A. elutus*. The provisional key to identification given in the Guide is based on the characters of the eggs of the different forms, but information on other characters is added in parentheses. It includes provisional information on the characters of the external spine of the harpago.

The external spine rises from the dorsal lobe, and when two spines are present on this lobe it is the spine which is situated most apically.

When an extra spine arises from the dorsal lobe a little below the apex, the external spine is spoken of as double.

The intermediate spine rises at some point between the dorsal lobe and the apical spine (ventral lobe). When near the apical spine it often gives the appearance of a doubling of that spine.

I have recently dissected out and examined the external spine of the harpago of the following forms. All the male insects used had been reared from eggs laid in the laboratory and identified by the examination of batches of these eggs.

A. elutus. The insects were reared from the egg stage by Mr D. J. Lewis in the laboratory at Valona, Albania.

A. maculipennis type. The insects were reared from the egg stage by Dr Chelarescu-Vieru at Socola, near Jassy, Northern Roumania. Number of specimens dissected and examined, 120.

A. maculipennis var. *atroparvus*. The insects were reared from eggs laid in the laboratory at Horton by gravid females collected in south-eastern England and from eggs laid by the genetically pure stock which has been maintained continuously in the Horton laboratory for more than a year. Number of specimens dissected and examined over 100.

A. maculipennis var. *labranchiae*. The insects were sent by Dr Hackett from Italy.

A. maculipennis var. *messeae*. The insects were reared from eggs laid in the laboratory at Horton by adults collected in England at Windsor, Exminster and Epsom.

In order to ascertain correctly the characters of the external spine of the harpago it is essential that the spines should be dissected completely from surrounding structures, stained, and rolled with pressure when they have been mounted under a cover-slip. The photographs which accompany this note are of spines treated in that way.

The conclusion at which I have arrived from the study is that, in general, the shape, length and other characters of the external spine of the harpago in most varieties are more constant than was at first supposed. The usual findings are stated in the following statement which may be helpful as a key to identification:

External spine blunt or round and of almost equal width throughout its length. Its length, which is a fairly constant character, is nearly two-thirds that of the apical spine ... **A. elutus**

External spine blunt or round and of almost equal width throughout its length. Its length varies greatly, being in some examples very short, in others as long as the apical spine **A. maculipennis** type

External spine always very sharp and tapering evenly from base to tip. The spine is nearly always double ... ... var. **atroparvus**

External spine always very sharp and tapering evenly from base to tip. The spine is usually single ... ... var. **labranchiae**

External spine short, medium, or long; sharp, blunt, or rounded. It tapers little from base to tip and when pointed it sharpens suddenly just below the tip ... ... var. **messeae**

It will be seen that the characters of the external spine vary most in the variety *messeae*. This variability would make it difficult to identify single specimens caught in nature. On the other hand, the difficulty would not arise when the problem is to identify the insects reared from a given batch of eggs because in that case the finding of great differences between the characters of the external spines of several males reared from the batch would indicate that *messeae* was the variety concerned. On the whole I think it is justifiable to conclude that as a general rule in batches of eggs with atypical surface patterns[1], the species could be determined accurately by dissecting out and examining the external spines of the harpagos of a number of male insects reared from them.

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[1] As many as 30 per cent. of batches of eggs may present an atypical surface pattern.

I. *ELUTUS*

Fig. 1.

Fig. 2.

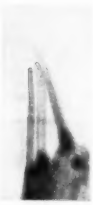

Fig. 3.

II. *TYPICUS*

Fig. 4.

Fig. 5.

Fig. 6.

III. *ATROPARVUS*

Fig. 7.

Fig. 8.

Fig. 9.

IV. *MESSEAE*

Fig. 10.

Fig. 11.

Fig. 12.

V. *LABRANCHIAE*

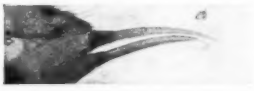

Fig. 13.

The harpago of varieties of *A. maculipennis*, showing characters of the external spine.

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EXPLANATION OF PLATE XIX

I. *A. elutus.*

Figs. 1 and 2. Round external spine; also with second spine on the dorsal lobe. No intermediate spine.

Fig. 3. An atypical specimen in which the external spine sharpens slightly towards the tip. Intermediate spine present.

II. *Type form.*

Fig. 4. Typical external spine. Possibly an intermediate spine is also present.

Fig. 5. The nearest to a pointed spine seen in this variety. Pressure ruptured the spine at its apex. No intermediate spine and no doubling of the external spine.

Fig. 6. Typical external spine. The bulbous appearance was caused by pressure on this spine.

III. Var. *atroparvus.*

Figs. 7, 8 and 9. Typical external spine; in all cases double.

IV. Var. *messeae.*

Fig. 10. Short semi-blunt external spine, triplicate and an intermediate spine.

Fig. 11. External spine sharply tipped and double.

Fig. 12. The external spine sharpens acutely just below the tip and is double. Also shows an additional spine midway between the apical and dorsal spine.

V. Var. *labranchiae.*

Fig. 13. External spine tapering slowly and becoming sharply tipped.

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RESEARCHES ON THE INTESTINAL PROTOZOA OF MONKEYS AND MAN

VII. ON THE *ENTEROMONAS* OF MACAQUES AND *EMBADOMONAS INTESTINALIS*

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> The Mind...has the peculiar and constant Error of being more moved and excited by Affirmatives, than by Negatives; whereas it should duly and equally yield to both.—F. Bacon (1620), *Nov. Org.* lib. I, aph. 46[1].

CONTENTS

	PAGE
Preface	564
A. *Enteromonas*	
(1) General	566
(2) Nomenclature	567
(3) Occurrence in Macaques	570
(4) Natural spread of infection among Macaques	571
(5) Duration of infections	573
(6) Cultivation	574
(7) Attempt to transmit *Enteromonas* from Macaque to Man	579
(8) Discussion	581
B. *Embadomonas*	
(1) General	582
(2) Nomenclature	583
(3) Cultivation	585
(4) Attempts to transmit *Embadomonas* from Man to Macaques	587
(5) Discussion	589
Summary and conclusions	590
References	590

Preface

This series of *Researches*[2] was undertaken in order to elucidate various problems concerning the intestinal Protozoa of Man with the aid of monkeys as experimental animals. It is not primarily intended as a survey of simian protozoa—a comparatively easy task which is now being pursued with more or less success by other workers, but one so vast that it cannot be completed in my lifetime.

[1] Bacon's own words were in Latin; but I give the excellent translation of Dr Peter Shaw (1733) because I have been upbraided by several friends in the past for quoting this language. Unfortunately Bacon himself left us no English version of his great work.

[2] See Dobell (1928–34) for the earlier instalments.

Up to the present, the monkeys which I have chiefly used have been macaques: and there is, at the moment, a general belief—perhaps well-founded, and partly due to my own publications—that all the intestinal protozoa living in these monkeys are specifically identical with similar forms found in men. I have already shown that *Entamoeba histolytica* and *Endolimax nana* naturally inhabit both Man and the Macaques, and that these amoebae are readily transmissible from one host to another: and I have shown, further, that an intestinal *Trichomonas* of *Macacus* is transmissible to Man, and have adduced evidence to prove that the intestinal and vaginal forms of these flagellates are the same in both hosts. Unpublished—or partly published—researches along similar lines have led me to similar conclusions regarding several other species.

But it is still premature to conclude that all monkeys and all men harbour identical species of intestinal protozoa. The difficulty of proving the identity of any species living in Man with a similar form inhabiting a monkey was well brought out in my study of the trichomonads of macaques[1]; and in the present instalment I shall record further difficulties which I have encountered in my experiments with two other flagellates—*Enteromonas* and *Embadomonas*. The study of these small organisms is beset with all manner of snares, and I now give my own experiences partly as a caution to others and partly because I believe that my experiments are not likely to be repeated, with equal safeguards and equally good material, by any other worker in the near future. Few other workers, indeed, are ever likely to have the simultaneous desire, opportunity, and energy, to carry out comparable experiments, which have been possible in my own case only because I was able to perform them concurrently with other—apparently more profitable—investigations.

In anticipation of what I now have to relate, I may say at once that the main object of the present researches was to demonstrate—by experimental transmission from one host to another—that the *Enteromonas* and *Embadomonas* of Man and *Macacus* are specifically identical; but I have failed to do so. All my experiments were entirely negative.

Nevertheless, negative experiments are not necessarily negligible; and I have Bacon's authority for emphasizing their importance. I give my own for what they may be worth. Although they are negative, they yield a small amount of exact information which is not yet obtainable elsewhere.

In the course of this work I have been able to throw some light on several subsidiary problems which are still unsolved. Since 1921 I have written nothing about the disputed species "*Enteromonas hominis*" and "*Embadomonas intestinalis*" of Man, but during this interval new information has been obtained and published by other workers. I believe—after reading much, if not all, that has appeared in print, and continuing my own studies uninterruptedly—that the nomenclature, at least, of both these flagellates should no longer be in doubt. I therefore think it worth while to publish my own conclusions now for the information of other students. These conclusions are

[1] Cf. Dobell (1934).

admittedly not final—all my researches being still in active progress—so I once more beg all readers to bear this in mind. I claim no finality for my present or previous results.

A. *Enteromonas*

(1) *General*

The following observations and experiments are concerned with a small flagellate living in the large intestines of macaques, but otherwise indistinguishable from *Tricercomonas intestinalis* (of Man) as described by Wenyon and O'Connor in 1917 or *Enteromonas hominis* as defined by me[1] in 1921. I shall consider its nomenclature immediately, but must first note a few particulars about the organisms themselves, as these data are essential for understanding most of what follows.

Enteromonas hominis (as here understood) is a minute flagellate some 4–10μ in length and 3–6μ in breadth. It possesses a single vesicular nucleus, situated anteriorly, and 4 flagella arising from blepharoplasts contiguous with its anterior pole. Of these flagella, 3 are free and directed forwards; the fourth is directed backwards, and lies within or attached to the surface of the body until it terminates freely near the hind end. No other permanent organs are present: there is no mouth, undulating membrane, or axostyle (unless the endocytoplasmic part of the posterior flagellum or "axoneme" be so interpreted).

Multiplication occurs by longitudinal division into two, and sometimes by multiple fission[2] (into 3 to about 8 daughter-individuals): but it is characteristic of this flagellate that division is often incompletely achieved. Double individuals—with 2 nuclei, 2 sets of anterior flagella, and 2 axonemes terminating in posterior flagella—are very frequently found in faeces, in intestinal contents, and in cultures. They may long remain in this "diplozoic" condition, and closely resemble flagellates of the genus *Hexamita* (= *Octomitus*).

The cysts of *E. hominis* are characteristic. They are elongate oval structures, about 6–8μ in length and 4–5μ in greatest width. When first formed each cyst contains only a single nucleus; but this afterwards divides twice, so that the ripe cyst is quadrinucleate—the 4 tiny nuclei lying in pairs at opposite poles.

Since the original descriptions of this organism were published, many other accounts of it—under various names—have appeared. The best is that of Boeck (1924), who called it *Tricercomonas intestinalis* and was the first to cultivate it continuously *in vitro*. Even his description, however, is incomplete, while his views on the nomenclature are now demonstrably incorrect. But I shall consider this question in the following subsection, and wish merely to emphasize here that the *Enteromonas* of Macaques—which I am about to discuss particularly—is morphologically identical in every way with *E. hominis* as just described. I have not thought it necessary to give any pictures

[1] See Dobell and O'Connor (1921).

[2] Incompletely described by Fonseca (1927), Cunha and Muniz (1927*a*, 1929), and several other authors.

of the simian forms, as those from Man have already been frequently depicted elsewhere[1].

I may note at this point that I have had another opportunity of studying the *Enteromonas* of Man since I attempted to define it in 1921. In 1924 a distinguished Italian colleague asked me to examine him, as he suspected an infection with *Entamoeba histolytica*—having found "amoebae" in his stools, and having suffered for many years from "colitis". On examination I found that his amoebae were really *E. coli*; but I also found that he was infected with *Enteromonas*, and I was able to obtain excellent living and permanent preparations of this flagellate from his faeces. I have used this material especially—in addition to that previously obtained—for comparison with my findings in macaques.

(2) *Nomenclature*

In an earlier publication[2] I discussed the nomenclature and systematic status of the genus *Enteromonas*, and I there pointed out that *Enteromonas* da Fonseca, 1915, and *Tricercomonas* Wenyon et O'Connor, 1917, are probably synonymous. At that time (1921), however, the data were admittedly insufficient to demonstrate my inference conclusively, and it was not generally accepted. A few workers, it is true, were impressed by my arguments, but most were not; while several said my conclusions were "unjustified" or "unjustifiable", and at least three accused me of merely "creating confusion" —two of them even alleging that there was "no evidence" for my view. But as my evidence was carefully collected, sifted, and recorded, and can still be read by anyone interested in this matter, there is no need to answer such "criticisms".

Apart from Brug (1923), who found my identification of *Enteromonas* with *Tricercomonas* "attractive", and provisionally accepted it, almost every author[3] rejected my conclusion without duly considering the grounds upon which it was based. But during the last few years two independent papers have appeared in support of my temerarious opinion. Schourenkova and Demina (1929) studied, at Moscow, a case of "*Tricercomonas*" infection which had features leading them to believe that this flagellate must be identical with *Enteromonas*: while quite recently López-Neyra and Suárez Peregrin (1934) have reached the same conclusion after studying no less than 58 similar cases at Granada. The observations of these two Russian ladies and these two Spanish gentlemen are in complete accord with my earlier inference. Nevertheless, their own conclusions are based upon identical premisses: they merely multiply the instances from which my deduction was drawn, without adding any data

[1] Cf. Dobell and O'Connor (1921, Pl. V, figs. 62–68), etc.

[2] Dobell and O'Connor (1921). As there explicitly stated (p. 80), Dr O'Connor was not responsible for any of the opinions—on this subject—which I then expressed. Since I cited all the relevant literature fully in this publication, I now give no specific references to most of the earlier works.

[3] See, for example, Lynch (1922), Cunha and Pacheco (1923), Boeck (1924), Thomson and Robertson (1925), Wenyon (1926), Fonseca (1927).

essentially different or new. Consequently, while I regard their work as confirmatory, I cannot regard it as a final solution of the main problem—whether the *Enteromonas* of Fonseca (1915) is, or is not, identical with the *Tricercomonas* of Wenyon and O'Connor (1917). This riddle can only be solved finally by comparison of the original type-specimens.

At the time (1921) when I expressed the opinion that Fonseca's *Enteromonas* was probably identical with the *Tricercomonas* of Wenyon and O'Connor, I had been unable to examine Fonseca's original preparations. His type-specimens were unobtainable, as also were those of Chalmers and Pekkola—which then seemed important for the decision of various minor details. In order to determine the identity or diversity of the organisms involved, it was obviously necessary to compare all the original types, but only those of Wenyon and O'Connor were accessible to me[1]. Fortunately, however, Wenyon was afterwards able to examine the original preparations of Chalmers and Pekkola, and has now reported his findings (Wenyon, 1926); while in 1929 he even succeeded in obtaining Fonseca's "type-specimen" of *Enteromonas hominis*, with several other important preparations, from the Brazilian investigator himself. Dr Wenyon kindly lent me these slides, so that I was able to study them carefully. We examined and discussed them together in April 1929, and I shall now record my own findings.

I must recall that Fonseca first described and named *Enteromonas* in 1915, and subsequently redescribed it—with variations—on several occasions, after studying further material[2]. All his accounts are of the flagellate forms alone: he has never yet described its cysts. In his most recent paper (Fonseca, 1927) he repudiates my identification of his flagellate with the *Tricercomonas* of Wenyon and O'Connor, and says that the existence of his genus as a separate entity has been confirmed by a Japanese worker, Dr I. Tochihara, who had been misled by my statements. Fonseca (1927) briefly records Tochihara's discoveries[3], but in doing so again makes no mention of cysts. The discovery of the cysts of the original *Enteromonas*, however, would be of supreme importance: for those of *Tricercomonas* were described by Wenyon and O'Connor, and are so characteristic that they are diagnostic of the genus.

Fonseca's preparations which I have now examined are 3 in number:

(1) His type-specimen (marked "*lamina typo*", and dated Rio, 5. vi. 1915).

(2) A specimen from his "second case" (dated 13. x. 1921).

(3) A slide made by Tochihara, in Japan, and guaranteed to contain the same organism.

[1] I may note—as I have been publicly blamed for not examining the type-specimens—that two requests which I made to Dr Fonseca for the loan of his original slides elicited no response. Dr Chalmers, however, whom I knew personally, in answer to my repeated petitions promised several times to send me his preparations "as soon as possible". Nevertheless, up to the time of his lamented death (1920) he never did so, and I have still had no opportunity of examining any of his specimens: but they are of no material importance for the present discussion.

[2] Cf. Dobell and O'Connor (1921).

[3] I have been unable to find any account of his findings published by Tochihara himself.

Although the first two slides were poor, and apparently much faded, it was still possible in 1929 to identify some of the flagellates in them. In (1) I found a few undoubted specimens of *Tricercomonas*, and no other recognizable protozoa. No cysts were discoverable. In (2), however, both flagellate forms and cysts of *Tricercomonas* were identifiable, but also flagellates and cysts of *Embadomonas*. Finally, (3)—a much better preparation—contained many typical *Tricercomonas* flagellates and a few cysts of the same organism, but no other species[1].

Since the type-specimen of *Enteromonas*, and 2 others guaranteed by the author of the genus to be identical, all contain the same organism as that named *Tricercomonas* subsequently, it is obvious that these genera must be synonymous.

Dr Wenyon, I know, concurs in this opinion: and though he has not personally published any statement to this effect, his view has been made public by Mac Arthur (1930)[2] and also by Fonseca (1932) in a more recent work not generally accessible. And apparently even Fonseca himself is now willing to admit that his original *Enteromonas* is identical with *Tricercomonas*, and that their seeming difference was due merely to his own inexact descriptions[3].

Consequently, there can no longer be any room for doubt that *Enteromonas hominis* Fonseca, 1915, and *Tricercomonas intestinalis* Wenyon et O'Connor, 1917, are two names for the same organism; and that the former, having priority, is the correct designation. The proposal of Schourenkova and Demina (1929)—who are in agreement regarding the identity of the flagellates themselves—to adopt the name "*Tricercomonas hominis*" is contrary to the Rules of Nomenclature, and therefore cannot be accepted (as already pointed out by López-Neyra and Peregrin).

I drew my conclusion tentatively in 1921 from the uncertain data then available, and it has now been completely justified. It was not a mere guess, based on no evidence; and if—as several authors allege—my deduction "created confusion", I was not personally responsible for it. At any rate, the essential facts are now all on record, and future writers should therefore be able to pronounce an equitable judgement. For my part, I have no hesitation in using, in the following pages, the generic name *Enteromonas* for all flagellates morphologically identical with "*Tricercomonas intestinalis*"—whether they were found in Man or in monkeys of the genus *Macacus* [=*Silenus*] or in any other hosts.

[1] *Dientamoeba fragilis* was, however, also present in this preparation.

[2] *Op. cit.* p. 114 note: "Wenyon points out, from an examination of the original preparations, that, as Dobell conjectured, *Tricercomonas* is a synonym of *Enteromonas*."

[3] Cf. Fonseca (1932), pp. 29–30. On p. 30 he says: "Hoje tendemos para admittir como verdadeira a hypothese de Dobell e O'Connor de que *Enteromonas hominis* e *Tricercomonas intestinalis* sejam um mesmo parasito e que, em nossa observação inicial, nos tenha passada despercebida a existencia do flagello recurrente intraplasmatico...." I am greatly indebted to Dr C. M. Wenyon not only for calling my attention to this publication, but also for lending me a copy. Without his help I could have had no knowledge of it, as it is not generally available to students in Europe.

I must add that the organism described as "*Octomitus pitheci* n.sp." by da Cunha and Muniz (1929*a*) from *M. rhesus*—imported into Brazil—appears to me to be merely a misinterpretation of the "diplozoic" forms of *Enteromonas*. In spite of obvious discrepancies in his description, I also regard it as probable that the "*Hexamita*" which Wenrich (1933) has more recently recorded from the same host (1 monkey, studied at Philadelphia) should be interpreted similarly. At all events, I am certain—from my own observations—that *Enteromonas* occurs commonly in *M. rhesus*; and that double individuals, closely resembling *Hexamita*, are frequently present in the faeces of this macaque. Consequently, until more satisfactory accounts of the "*Hexamita*" and "*Octomitus*" of *M. rhesus* are forthcoming, with exact data for distinguishing these organisms from the diplozoic forms of *Enteromonas* occurring in the same situation, I must continue to regard *Octomitus pitheci* Cunha et Muniz, 1929 [=*O. pithici* Wenrich], and the *Hexamita* of Wenrich (1933), as synonymous with *Enteromonas*.

Similar considerations are applicable to the "*Octomitus*" which has more than once been described from Man: and I think it probable that the "new species"[1]—"*Octomitus rastegaiev*"—recently recorded by Perekropoff and Stepanoff (1932) is, in part at least, merely a misidentified *Enteromonas*. To the best of my belief there is still no conclusive evidence to prove that a true *Hexamita* (or *Octomitus*) lives naturally in Man or any other member of the Primates.

(3) *Occurrence in Macaques*

It seems to me probable that *Enteromonas* occurs commonly in the intestines of macaques: for without making special search I have found it living in 3 different species, and I have no reason to suppose that my findings—though almost accidental—are exceptional.

I first found enteromonads in *M. sinicus* in 1924, and this was recorded in the annual *Report of the Medical Research Council* for 1924–5. Although I referred to this again, and to some later findings, a few years afterwards (Dobell, 1928), Thomson (1929) felt justified in stating subsequently that "*Tricercomonas* has not been found in monkeys". Similar statements have been made by other writers, but I can only suppose that they were due to oversight. *Enteromonas* probably occurs frequently in *Macacus*; yet Kessel (1928) studied a score of macaques (4 species) in China and never once observed this flagellate[2], while Hegner and Chu (1930), who examined 44 wild macaques in the Philippines, merely say that they found "a number of small flagellates... that resembled *Tricercomonas intestinalis*, and *Hexamita*" but made no proper study of them. I have little doubt, however, that some of their forms resembling

[1] The species—if valid—is named in an unorthodox manner. Its name, as published, is not in accord with the accepted international rules and recommendations of zoological nomenclature.

[2] It is just possible that the small biflagellate organism which he found and identified as *Bodo edax* Klebs was really *Enteromonas*. At all events, it was not *B. edax* or even—to judge from his figure—a *Bodo* at all. Further information about this form is needed.

Tricercomonas and *Hexamita* were really *Enteromonas*; and consequently, that careful inquiry will show that this flagellate occurs in *M. philippinensis*.

My own records prove that *Enteromonas* occurs in macaques of more than one species. Though I have found it in only a few monkeys which came into my possession casually, I cannot believe that it is rare. Indeed, I believe it to be common in *M. rhesus*, at least, as I have already observed it not only in one of my own monkeys of this species but also in several others belonging to other workers in the Institute; and, as already noted[1], I believe that the "*Hexamita*" found in *M. rhesus* by da Cunha and Muniz (1929*a*) and Wenrich (1933) was probably the same flagellate.

Further observations are, however, obviously needed to determine the distribution of *Enteromonas* in the Macaques. All I can say at present, from my own knowledge, is that flagellates of this genus may occur "naturally" in captive specimens of *M. rhesus* [= *Macaca mulatta*], *M. sinicus* [= *radiatus*], and *M. nemestrinus*; and that they appear to be specifically identical in all these hosts, and are morphologically indistinguishable from *E. hominis*.

(4) *Natural spread of infection among Macaques*

As I have been able to make a few observations on the spontaneous spread of infection with *Enteromonas* among a small group of macaques, my records appear worth publishing—especially as no similar data are yet available for any other protozoa of any other monkeys. To make my findings clear I must first give a few exact dates and data, some of which have already been noted in other connexions.

My original family of captive macaques consisted of 4 individuals:

(1) **Polo** (*M. sinicus* ♂)
(2) **Jacko** (*M. rhesus* ♂)
(3) **Mungo** (*M. sinicus* ♂)
(4) **Susanna** (*M. sinicus* ♀)

All these animals were immature, and had been very recently imported by the dealer from whom they were obtained. They came to the National Institute in May 1924; and after being used for some negative (not protozoological) experiments by Dr P. P. Laidlaw, they were discarded by him at the end of June and came into my possession in July. They were then kept in the same room—isolated from all other monkeys, but allowed to play together and associate freely, though caged separately at nights and feeding-times.

In July 1924 I began to examine the faeces of these macaques microscopically, in order to discover all the intestinal protozoa which each individual harboured. I studied every animal in great detail, and found *Enteromonas* in only one. This was Mungo (*M. sinicus*), in whose faeces I first found a few cysts in a stained preparation at the fourth[2] examination (on 15. viii. 24). All the other monkeys appeared to be uninfected.

[1] See p. 570 *supra*.

[2] Previous examinations (7. viii. 24, 8. viii. 24, 12. viii. 24) were negative for *Enteromonas* and all other flagellates except *Giardia*.

In November 1924 I first began to study the protozoa in my macaques by cultural methods[1], and thereby confirmed the previous microscopical findings. *Enteromonas* developed in cultures made from Mungo's faeces, on numerous occasions, and from Mungo's faeces alone.

At the beginning of 1925—when I had, so far as I could determine, only one monkey infected with *Enteromonas*—I added a fifth member to my family. This was **Bonar** (*M. sinicus* ♂), who had then been in the Institute for over a year, and had been kept by his owner, Dr W. Mair, separately caged in a small room with 2 other macaques (**Samson** and **Delilah**, both *M. rhesus*). I examined all three of these monkeys carefully and could find *Enteromonas* in none of them.

Accordingly, by February 1925 I had obtained 5 macaques for study (4 *M. sinicus* and 1 *M. rhesus*), but only 1 of them (Mungo, *M. sinicus*) appeared to be infected with *Enteromonas*. I continued to examine them all, at frequent but irregular intervals, and to investigate all their intestinal protozoa by every means available. I imagined that they would all soon acquire one another's various infections; but in this I was mistaken. Yet they all became infected, finally, with *Enteromonas*, which they must—almost certainly—have acquired from Mungo. The evidence is, from the nature of the case, inconclusive: but it is, to my mind, very cogent. I should add that, at the time of these observations, none of my macaques harboured any other intestinal flagellates which could be confused with *Enteromonas*. The only other flagellate present—in several monkeys—was *Giardia*, which has never yet been cultivated, and never lives for more than a few hours in cultures made from faeces. On the other hand, *Enteromonas* develops with great regularity in primary cultures of the Boeck-Drbohlav type, so that its presence can usually be detected readily in any infected macaque or man by cultural examination.

My monkeys were not examined daily, so I am unable to state the exact date on which the infection first manifested itself in any particular animal. The dates on which I first detected their infections were as follows:

Susanna	was	found	infected	on	21. vii. 25.
Jacko	,,	,,	,,	,,	19. viii. 25.
Polo	,,	,,	,,	,,	31. viii. 25.
Bonar	,,	,,	,,	,,	8. ix. 25.

These observations seem to show that the *Enteromonas* of *M. sinicus* can not only spread by contact from one macaque to another of the same species, but also to *M. rhesus*: but I must give more exact data in support of this conclusion, and select Susanna and Jacko as the best examples.

Susanna (*M. sinicus*), first found infected on 21. vii. 25, was examined for about a year previously with consistently negative results. I examined her faeces microscopically on 52 occasions during this period, and culturally more than 10 times—7 of them being exhaustive, and made in the course of prolonged

[1] As a result of the discoveries of Boeck and Drbohlav, acknowledged more fully in earlier instalments.

attempts to isolate amoebae[1]. No flagellates of any sort were ever found at any examination before 21. vii. 25: but since that date *Enteromonas* has been found more often than not in fresh faecal specimens, and in cultures made from them it has developed almost invariably. Since the date when her infection was discovered, I have made some hundreds of positive examinations, and the infection is still easily demonstrable—both microscopically and culturally. I have thus no doubt that Susanna acquired her infection "naturally" from another monkey of the same species.

Jacko (*M. rhesus*), first found infected on 19. viii. 25, was also studied for about a year previously with negative results. During this period I examined his faeces 30 times microscopically, and in great detail by cultural methods on various occasions[2]—always with completely negative results. After I discovered his infection it was always readily demonstrable until the day of his death (15. ii. 28) about 2½ years later. During the whole of this period *Enteromonas* was frequently found in his faeces microscopically, and developed in cultures made from them almost always. It thus seems certain that Jacko acquired his infection from one or other of his companions (Mungo, Susanna, or Polo): and consequently, that *M. rhesus* can acquire an infection with the *Enteromonas* of *M. sinicus* by contact with infected individuals of this species.

The evidence relating to the other monkeys (Polo and Bonar—both *M. sinicus*) is similar but less certain, for I examined them less frequently. But it seems equally clear, and I have little doubt that both acquired infection through contact with their infected playmates.

Rosa (*M. rhesus*) was added to my family in June 1927, but had been carefully studied by Miss A. Bishop since October 1926. This monkey was found to be naturally infected with *Enteromonas*, and her infection has persisted ever since. I have made some hundreds of examinations of her faeces during the last 8 years, and have very seldom failed to recover the flagellates in primary cultures.

Susanna (who acquired her infection through contact with another macaque of the same species) and Rosa (naturally infected) have now been closely associated for some 7 years; but during this period they have never been in contact with any other monkey whatsoever, and I have taken every precaution to preclude their accidental infection with intestinal protozoa of every sort.

(5) *Duration of infections*

I have now collected a few data—not previously recorded by any observer—which indicate the length of time for which infections with *Enteromonas* may persist in macaques. Briefly, my observations show that when once an infection has been acquired it endures indefinitely. All my monkeys have retained

[1] Susanna harboured *E. histolytica*, *E. coli*, and *Endolimax nana*—all of which I finally succeeded in isolating in pure culture.

[2] I was attempting, at the time, to isolate strains of *E. histolytica* from this monkey; and latterly, to prove that he was uninfected with *E. nana* and *Trichomonas*. Cf. Dobell (1931, 1933, 1934).

their infections for the entire period of observation: no infection has ever been found to die out spontaneously.

I have exact records for 6 macaques—4 males and 2 females, all very carefully studied for several years at least—and my findings are briefly summarized in the accompanying Table I.

Table I. *Duration of infections with* Enteromonas *in Macaques*

Host	Observed duration	Remarks
Polo (*M. sinicus*)	3 years (Aug. 1925–July 1928)	Infection acquired Monkey killed 28. vii. 28
Jacko (*M. rhesus*)	2½ years (Aug. 1925–Feb. 1928)	Infection acquired Killed 15. ii. 28
Mungo (*M. sinicus*)	4 years (Aug. 1924–Aug. 1928)	Natural infection Killed 8. viii. 28
Susanna (*M. sinicus*)	10 years (July 1925–May 1935)	Infection acquired. Still alive and infected
Rosa (*M. rhesus*)	8½ years (Nov. 1926–May 1935)	Natural infection. Still alive and infected
Bonar (*M. sinicus*)	2 years (Sept. 1925–Sept. 1927)	Infection acquired Killed 8. ix. 27

No data are as yet available for determining the duration of *E. hominis* infections in Man. It may be recalled, however, that Wenyon and O'Connor (1917) believed that in human beings infection is sometimes only transient—persisting for not more than a week or two: but their cases were not followed up for times sufficient to demonstrate this conclusion, and no confirmatory observations have since been published. Though I have not myself been able to study any human infection for more than a few days, I find no reason to believe that *Enteromonas* is usually a less persistent inhabitant of men than it is of macaques.

(6) *Cultivation*

Enteromonas hominis—under the name of *Tricercomonas intestinalis*—was apparently first obtained in "cultures" by Lynch (1922). "The organism grew well in ascitic fluid diluted with 4 parts of 0·9 per cent. sodium chloride solution at 37° C. for four days but transplants were not successful." The first really successful and propagable cultures, however, were made by Boeck (1924)[1], who used a solid-liquid medium composed of coagulated egg covered by human serum diluted with Locke's solution. Media of this type are now in general use for the cultivation of intestinal protozoa, and it was with a modification of Boeck's methods that I first succeeded in cultivating, and isolating in "pure" culture, the *Enteromonas* of macaques.

The *Enteromonas* of *Macacus sinicus*, *M. rhesus*, and *M. nemestrinus* can be readily grown in any of the solid-liquid media used for cultivation of *Entamoeba histolytica*[2]. I generally employ those designated "Ehs" (inspissated

[1] *E. hominis* (from Man) has since been cultivated, by similar methods, by many workers (Thomson and Robertson, 1925*a*; Knowles and Das Gupta, 1929; Schourenkova and Demina, 1929; Svensson, 1934; etc.).

[2] See Dobell and Laidlaw (1926*a*), p. 294.

whole egg, covered by dilute horse-serum) and "HSre" (inspissated horse-serum, covered by dilute egg-white). In my media Ringer's solution is purposely used instead of Locke's as a diluent, because dextrose is an unnecessary and sometimes unfavourable constituent[1]. The addition of solid rice-starch is also unnecessary, as *Enteromonas* cannot ingest it.

Primary cultures, inoculated with the fresh faeces of an infected macaque and incubated for 24 hours at 37–38° C., almost invariably show an abundant growth of *Enteromonas*. The flagellates may persist and increase for several days, and may sometimes even encyst: but in subcultures they seldom develop well, and often not at all, though *Blastocystis* and other concomitant organisms grow luxuriantly. Consequently, it is extremely difficult to obtain a pure strain of *Enteromonas*—free from all other protozoa, and cultivable *ad libitum*[2]. Moreover, a pure strain, when obtained, is of little use for experimental purposes unless it will complete its life-cycle (*i.e.* form cysts) *in vitro*.

Although I have made scores of attempts during the last 10 years to obtain a pure and freely encysting strain of *Enteromonas* for experimental study, I have only once succeeded. The success—as in all such attempts—was partly accidental: for it depends largely upon the bacteria (unknown and unpredictable) which happen to be present in the initial sample of faeces, and upon the fortuitous survival in subcultures of the flagellates *together with those particular bacteria which are essential for their complete development.*

As I was able to isolate a pure and encysting strain (*ME.*) of *Enteromonas* in 1926, and used it for an experiment described in the next subsection, I may add a few further particulars.

Isolation of Strain ME.—The origin of this strain was a loose stool passed by my monkey Mungo (*M. sinicus* = *radiatus*) on 9. iii. 26. At this date, I was examining his faeces daily in the course of an attempt to infect him experimentally with *Trichomonas*[3]. He was known to be naturally infected with *Entamoeba histolytica* (2 strains, with large cysts and small), *Endolimax*, *Giardia*, and *Enteromonas*. In the primary culture (in "HSre + S." medium) all these protozoa except *Giardia* developed as usual—along with the inevitable *Blastocystis* (but no *Trichomonas*). After making subcultures every day for the next 3 weeks, in the same medium, I obtained (on 31. iii. 26, in "Ehs" medium) an excellent mixed culture of *Enteromonas* and the small strain of *E. histolytica*. The large strain of this species, *Endolimax*, and *Blastocystis* were by then all

[1] A small amount of dextrose is naturally present, of course, in all these media: but the addition of more usually favours the growth of bacteria, which, by forming acid from it, retard or inhibit the development of concomitant protozoa.

[2] I may recall that Boeck (1924), who first cultivated *E. hominis*, never succeeded in freeing his cultures from *Blastocystis*, which gradually overgrew the flagellates and killed them off after 3 weeks. Thomson and Robertson (1925*a*) also suffered from *Blastocystis*, but say that they kept their cultures going by adding boiled faeces to them—they do not say for how long. As a general method I have found this worthless.

[3] See Dobell (1934), p. 547. As there recorded, the experiment with *Trichomonas* gave a negative result.

completely eliminated. I spent a good part of the following 3 weeks trying to separate the flagellates from the amoebae, and employed nearly every expedient I could think of. I finally succeeded by adopting the following device:

I found that when the mixed cultures were transplanted into "Ehs" medium, they always gave a good growth of both organisms, but their development did not run parallel. *Enteromonas* grew more rapidly than the amoebae, so that after incubation for only 24 hours it was often the only organism discoverable: whereas, after 2, 3, or 4 days, both protozoa were plentiful—the amoebae predominating latterly. Moreover, the amoebae crawled on the solid surfaces of the medium—on the egg and on the surface of the glass; while the flagellates often swam freely in the liquid at the bottom of the culture-tubes. I therefore made a series of rapid subcultures—every 24 hours—using as inoculum only a small amount of liquid (not sediment, if possible) from the bottom of each tube: and although amoebae subsequently developed in all the earlier cultures, after about half-a-dozen generations in this way I found they had been completely eliminated[1]. On 26. iv. 26—nearly 7 weeks after making the primary culture—I obtained a strain of *Enteromonas* (designated *ME.*) which I had at last proved to be "pure". No amoebae or other protozoa were discoverable in it by microscopical or cultural tests of any sort.

Although the first really "pure" culture obtained was of only the 26th generation serially, I had to make over 100 cultures in the process of isolating it—including the main line (as it turned out to be subsequently) and its collaterals. But as I succeeded at the same time in separating "pure" strains of both the large and the small races of *E. histolytica* present in the initial material, I had to make at least another 100 cultures in these parallel—in a sense control—experiments[2]. This will give the reader a rough idea of the labour actually needed to obtain a pure strain of *Enteromonas* from a macaque on one occasion when the attempt succeeded. I have made many similar attempts at other times, with more or less success: and I think it is no exaggeration to say, therefore, that if anyone wishes to repeat my experience he must be prepared to spend at least 2 months at the task and to make not less than 100 cultures.

All my efforts to separate *Enteromonas* from *E. histolytica* by cooling mixed cultures of free forms for various times, or by hatching cysts of the flagellates—which were often present—failed completely. Whenever I succeeded in obtaining a culture of *Enteromonas* by any other method, it was invariably found—sooner or later—to be contaminated with amoebae. But when once I obtained the flagellates alone, in "pure" culture, they grew splendidly. Every subculture, in many different media, showed a profuse growth, and in "Ehs" medium typical cysts were produced in abundance[3]. I thus had ample material

[1] I have recently (April–May 1935) succeeded in isolating *Enteromonas*, in pure culture, from a mixed culture with *Endolimax* by the same method. This strain (*RE.*) was derived from *M. rhesus*.

[2] I have kept full records, and can supply details, if necessary, to other workers along similar lines. At present I give approximate figures only.

[3] Previous workers—with *Enteromonas* from Man—appear to have been uniformly unsuccessful in obtaining a strain which would form cysts *in vitro*.

for study—for a time. Unfortunately, however, the flagellates gradually ceased to form cysts *in vitro*, and all my cultures finally contained nothing but flagellates. Every attempt to induce encystation failed completely: and as I could make no further experiments with flagellates alone, and had many other and more interesting experiments in hand at the time, I finally abandoned Strain *ME.* (2 parallel lines) on 18. v. 26. It had then been under cultivation for 30 generations—70 days altogether, but for only the last 3½ weeks in a pure state.

All my most accurate observations on the *Enteromonas* of macaques have been made on Strain *ME.* But they have been abundantly confirmed by a study of other strains, and of the flagellates and their cysts in faeces. I have cultivated numerous other strains from *M. sinicus* and *M. rhesus*—usually in an impure state—for various periods up to about 5 weeks: but they were all abandoned finally because I was unable to isolate the flagellates in "pure" culture, or because my "pure" strains—uncontaminated with any other protozoa or *Blastocystis*—refused to encyst. The following additional notes, therefore, while recording especially my experiences with Strain *ME.*, are of general application to the enteromonads in all my macaques.

Enteromonas, when accompanied by appropriate bacteria, grows excellently not only in many media of the solid-liquid (Boeck) type[1], but also in diluted serum alone (Barret's type of medium). I have obtained excellent cultures in 10 per cent. solutions of serum (horse, calf, or rabbit) in Ringer's fluid. In all these media the flagellates lived for 4–5 days, and often for a week: but I found it best to make subcultures every 4–6 days. Cysts were formed regularly—for a time—in solid-liquid media, but very seldom in liquid only. They usually appeared in cultures on the second day of incubation, and were most abundant on the third or fourth. On continued incubation, in the same medium, they invariably died without hatching—so far as I could determine.

I have made innumerable attempts to initiate cultures from cysts, but have usually failed to make them hatch *in vitro*. With Strain *ME.* I failed invariably, although the cysts in the inocula were often abundant and appeared to be ripe (4-nucleate), alive, and otherwise normal in every way after preservation in various liquids at room-temperature (*ca.* 15° C.) for any time up to about 3 weeks. Whenever I inoculated these cysts into culture-media—known to be favourable for the growth of the flagellates—and incubated them at 37° C., they died without showing any signs of development. Even cysts only 2, 3, 4, or 5 days old failed to hatch completely: while older samples—among which dead specimens were increasingly prevalent—appeared to degenerate even more rapidly on incubation.

I cannot account for this: I can only record my failure. On other occasions, with material from other sources, I have succeeded in obtaining cultures from

[1] Thomson and Robertson (1925*a*) could obtain cultures only in media to which they added faeces. In my experience such addition is neither necessary nor desirable. All my "pure" cultures were entirely free from human or simian excrement.

cysts (certainly unaccompanied by flagellates). I have done this not only with culture-cysts, but also with cysts obtained from faeces. But the positive results have been very few in proportion to the negatives, and have occurred under conditions which throw no light upon the factors concerned in excystation. As a rule it is easy to obtain cysts free from flagellates; because the latter are very sensitive to cold, while ripe cysts remain apparently unaltered for many days at ordinary room-temperatures or in the ice-chest. Flagellates in faeces, or in good cultures in various media, rapidly round up and become motionless and die when cooled. After exposure to temperatures of 10–20° C. for only a few hours, they generally appear dead; and when inoculated into suitable media, and incubated at 37° C., they cannot be recovered. I cannot give precise figures, however, because my findings are ambiguous. For instance, in several experiments I have obtained cultures from flagellates—all seemingly dead—kept at room-temperature for 24 hours: and in one instance after as long as 27 hours. But these were exceptions, and may possibly have been due to the presence of undetected cysts in the inoculum.

Drying appears to kill both flagellates and cysts instantly. Not only do they look dead after desiccation, but they also fail to develop when incubated in suitable media. I have made many negative experiments in this connexion, and the results seem conclusive.

Most of my successful attempts to obtain cultures from cysts have been achieved sporadically and unexpectedly. I may give the following as a good example: On 10. vii. 26 I concentrated and washed the cysts of *Enteromonas* (also of *E. histolytica* and *Endolimax*) present in great abundance on that date in the faeces of my monkey Bonar (*M. sinicus* ♂). I then suspended them in sterile distilled water, and kept them at room-temperature (*ca.* 20° C.). Every few days I inoculated samples of these cysts into suitable media—previously sown with bacteria known to be favourable for growth of the flagellates—and incubated them at 37° C. All the early cultures gave entirely negative results, although the cysts appeared to remain normal in the stock suspension. Those incubated on the 18th day (28. vii. 26), however, gave a copious growth of flagellates (accompanied by *E. histolytica*, whose cysts also hatched under the same conditions). But all later cultures—made on the 20th, 22nd, 24th, 26th, and 28th days—were again completely barren.

An experiment of this sort proves that *Enteromonas* cysts can survive at room-temperatures for 18 days, but supplies no other information. In similar experiments I have occasionally obtained good cultures from cysts kept for shorter periods[1], but never for longer; and I conclude, therefore, that they can live for at least 2½ weeks outside the body. Despite innumerable attempts I have not succeeded in ascertaining the precise conditions necessary for hatching.

From all my experiments and observations I conclude that flagellate forms of *Enteromonas* (from macaques) cannot usually survive for more than 24 hours

[1] For instance, in a similar series of cultures begun at about the same time (8. viii. 26), the only positive result was obtained with cysts kept in water for 6 days.

at ordinary room-temperatures (15–20° C.), though its cysts may remain viable—under similar conditions—for at least 2½ weeks.

Enteromonas is extremely resistant to emetine, both *in vivo* and *in vitro*. It can even withstand a concentration of 1 : 1000 in solid-liquid media[1], and has never been eradicated—or apparently affected—by doses of this alkaloid sufficient to cure infections with *E. histolytica* in my experimental macaques[2].

I may add here that I have usually had great difficulty in obtaining good permanent preparations of *Enteromonas*. The flagellates are comparatively easy to study when alive, but when fixed and stained they generally appear more or less shrunken and abnormal; while their internal structure and their flagella are often impossible to determine with precision—which accounts for the numerous divergent accounts already published. Strain *ME.*, when isolated pure, was a great disappointment: for the flagellates grew well only in association with intensely siderophilic bacteria, so that most individuals in stained preparations were impossible to interpret satisfactorily. In iron-haematoxylin films—in which minute details of flagellar and nuclear structure were alone demonstrable—most of the organisms were stuffed with deeply stained bacteria which completely obliterated the morphology of the flagellates themselves. I have found recently, however, in the course of experiments with another strain (*RE.*, from *M. rhesus*), that serviceable and even excellent preparations can be easily obtained by staining with Mallory's phosphotungstic-acid haematoxylin. In films fixed in Bouin's fluid, and stained progressively by this method, all details of nuclear and flagellar structure can be readily demonstrated.

(7) *Attempt to transmit* Enteromonas *from Macaque to Man*

I have always found it extremely difficult to isolate pure strains (*i.e.* free from all other protozoa) of *Enteromonas* from my infected macaques; and on the rare occasions when I have succeeded, the isolated strains have generally been worthless for any cross-infection experiments because they would not encyst. But, as noted in the previous section, I once obtained a pure strain which for a time produced cysts freely *in vitro*; and with this I was able to make a satisfactory attempt to infect a man.

The strain of *Enteromonas* was that just described (*ME.*), and the human subject was myself[3]. That I was uninfected with *Enteromonas* at the date of the experiment is certain. I made the attempt in April 1926; and my stools had then been consistently negative for this flagellate for nearly 20 years.

[1] The actual concentration of the alkaloid in such media is variable and uncertain (cf. Dobell and Laidlaw, 1926; Laidlaw, Dobell, and Bishop, 1928): but my experiments show that *Enteromonas* can survive in solutions at least 50 times as strong as those which are fatal to *E. histolytica* under similar conditions.

[2] Cf. Dobell with Bishop (1929), Dobell (1931). [3] Cf. Dobell (1933, p. 450; and 1934, p. 552).

Since the winter of 1906–7 I had examined them microscopically 221 times[1]; and from 14. viii. 25 until 22. iv. 26 I had made 30 careful and prolonged attempts—on various dates—to discover *Enteromonas* in myself by cultural methods. No flagellates of any kind—except *Giardia*, with which I was naturally infected, and which is still uncultivable by any known method—were ever found.

On 22. iv. 26 I ingested all the cysts in a "pure" culture of Strain *ME.* (*Enteromonas* from *M. sinicus*)—23rd serial subculture in "Ehs" medium. The culture itself was inoculated on 14. iv. 26, incubated at 37° C. for 2 days—when it was found to contain numerous flagellates and cysts (many ripe)—and was then kept at room-temperature for 6 days. I mixed all the sediment in the tube with a little sterile rice-starch, and then introduced the semiliquid mixture into 2 small gelatin capsules which I swallowed with a draught of water on an empty stomach. At the same time I inoculated a small amount of the sediment into a culture-tube, and put it in the incubator—to prove, if possible, that the cysts were viable: and I also fixed and stained a minute sample—to make sure that they were structurally normal.

The cysts were kept at room-temperature before ingestion not only to kill off all the flagellates originally present, but also as a safeguard against accidental contamination with *E. histolytica*. As already noted[2], Strain *ME.* was originally accompanied by a small strain of this amoeba; and I was not absolutely certain, at the date of the experiment, that it had been completely eliminated[3]. However, no amoebae were discoverable in the subculture made from the infective material, and none in the control-preparation fixed and stained at the same time. I am confident, therefore, that the material contained living cysts of *Enteromonas* and no other protozoa. Unfortunately, the subculture produced no flagellates: the cysts merely died on incubation. Had they hatched, it would have afforded additional evidence of their viability, but the fact that they did not do so means nothing, as I have rarely been able to obtain cultures from cysts of this species[4]. I may recall, further, that I have proved by experiment that the cysts of *Enteromonas* can live for far longer than 6 days at ordinary room-temperatures[5], so that I have no reason to believe that those used on the present occasion were not alive. They appeared, on the contrary, to be normal in every way.

After I swallowed the cysts I examined my stools with great care, both microscopically and culturally, every day for the next 16 days, and then every other day for a further 10 days[6]. Since then I have examined myself frequently,

[1] This number is minimal: it is derived from the examinations which I recorded in my notebooks. Actually I examined myself more frequently, but often failed—in early days—to keep any record of negative findings as I did not then realize their future value. [2] Cf. p. 576 *supra*.

[3] That my precautions were justified is shown by the fact that *E. histolytica* (small form) recurred in several parallel cultures—believed to be pure—made at the same period.

[4] Cf. p. 577 *supra*. [5] Cf. p. 578 *supra*.

[6] Many of these examinations were made under conditions of extreme difficulty, owing to the General Strike which occurred at this time. Nevertheless, I can guarantee their accuracy.

at irregular intervals, in the course of other experiments. All examinations have been consistently negative for *Enteromonas*. I have made altogether—up to the time of writing (May 1935)—281 negative microscopical and 275 negative cultural examinations during the 9 years following my attempt to infect myself experimentally in 1926.

The experiment was attended by no untoward results of any sort. I suffered no harm from the cysts and bacteria which I ingested, and feel satisfied by the evidence that I am not personally infectible with the *Enteromonas* (Strain *ME.*) of *M. sinicus*.

(8) *Discussion*

Although I am now only concerned with the species of *Enteromonas* occurring in Man and the Macaques, it is necessary to note that other species of the same genus—from other hosts—have already been described and named. Sometimes they have been referred to *Enteromonas*, and sometimes to *Tricercomonas*: and while it is now certain that these names are synonyms, it does not follow that all organisms recorded under either name are co-generic. Unfortunately, moreover, most students of such flagellates hitherto have not only made and recorded their own observations imperfectly, but they have often alluded to one another's publications with disconcerting inaccuracy.

The chief "species" of *Enteromonas* [=*Tricercomonas*] already reported are enumerated in Table II.

Table II. *Recorded species of* Enteromonas [=Tricercomonas]

Species	Recorder	Host	Locality
(1) *E. intestinalis*	Fonseca, 1918	Rabbit (*Oryctolagus cuniculus*)	Brazil
(2) *E. caviae*	Lynch, 1922	Guinea-pig (*Cavia cobaya*)	U.S.A.
(3) *E. fonsecai*	Yakimoff, 1923	*Ditto*	Russia
(4) *E. lagostomi*	Cunha and Muniz, 1927	Viscacha (*Lagostomus trichodactylus*)	Brazil
(5) *E. wenyoni*	Cunha and Muniz, 1927*b*	Opossum (*Didelphys aurita*)	Brazil
(6) *T. suis*	Knowles and Das Gupta, 1929	Pig (*Sus scrofa domestica*)	India

Some of the records—*e.g.* that of Yakimoff (1923)—are doubtful in the extreme: others appear to describe distinct species (for example, "*T.*" *suis*). After reading all the descriptions, however, I am still unable to reach any definite conclusions regarding their validity. All the organisms designated in the table are clearly *species inquirendae*; and it is impossible, at present, to ascertain how many of them are "good" species, or whether any of them are identical with *E. hominis* and the similar forms living in macaques. Many more accurate data are needed to clarify the present confusion.

Nevertheless, I am now satisfied that only one species of *Enteromonas* [=*Tricercomonas*] has yet been proved to inhabit the intestine of Man. Discrepancies in the various descriptions of it are manifestly due to malobserva-

tion or misinterpretation. The flagellates themselves are minute, and difficult to study, so that misdescriptions are to be expected.

The one definite human species of *Enteromonas* appears to have a world-wide distribution: at all events, it has already been reported from Man in Egypt, England, Italy, Sweden, Spain, Russia, India, Malaya, Japan, Brazil, and the United States—to mention only a few well-known and authentic records.

Of the distribution of *Enteromonas* in monkeys we still know very little. Flagellates of this genus certainly occur—and are probably common—in macaques of the species *M. rhesus*, *M. sinicus*, and *M. nemestrinus*. In all these hosts they appear to be identical. No cross-infection experiments have yet been made, though the observations recorded earlier[1] indicate that the *Enteromonas* of *M. sinicus* is readily transmissible to *M. rhesus*.

So far as my own observations and experiments go, they lead me to believe that the *Enteromonas* of Man is identical with that of the Macaques. I have been unable to discover any morphological or cultural character by which they can be distinguished: but I have also been unable to verify this interpretation by infecting a man experimentally with *Enteromonas* derived from a macaque, or *vice versa*. My one attempt was unsuccessful.

But a single negative experiment of this sort is obviously inconclusive: it proves merely that one man (myself) was uninfectible, under the conditions defined, with one strain of *Enteromonas* from one *M. sinicus*. In more favourable circumstances—with other strains of flagellates, other monkeys, and other men—the result might well have been positive[2].

On the whole, therefore, I am not convinced that the *Enteromonas* of macaques is specifically different from *E. hominis*. I believe, indeed, that future work will show that these species are identical—notwithstanding my failure to transfer the simian species to a man. In all other respects they are so closely alike that I attribute my lack of success to the use of unsuitable material or an uninfectible or insusceptible host.

B. *Embadomonas*

(1) *General*

The flagellates which I shall here call *Embadomonas* are minute organisms possessing 2 anterior flagella and forming characteristic uninucleate cysts shaped like sharply pointed eggs or lemons. *E. intestinalis* was first described by Wenyon and O'Connor (1917), and is now a familiar inhabitant of the human gut[3]. Its nomenclature will be discussed in the next subsection, while lists of the species hitherto referred to the genus have been given already by Bishop

[1] See p. 571 *supra*.

[2] Cf. my experiments with *Trichomonas*—already reported in an earlier instalment (Dobell, 1934).

[3] Cf. Dobell and O'Connor (1921), pp. 78–80.

(1931) and Wenrich (1932). The fullest description of any form living in a non-primate host is that by Collier and Boeck (1926) of *E. cuniculi* from rabbits.

Although *E. intestinalis* is now so well known, very little indeed is known about the similar flagellates which occur in monkeys. The first of these was recorded, under the name of *Waskia wenyoni*, by Fonseca (1917[1]) from a platyrrhine—*Cebus caraya*—in Brazil. The only available records relating to macaques are those of Kessel (1928), Hegner and Chu (1930), and Wenrich (1933).

Kessel (1928) states that he found "*E. intestinalis*"—assumed to be identical with the species in Man—in 1 specimen of *M. rhesus* (out of 8 examined) and in 1 *M. sanctijohannis* (out of 2), studied at Peking: but apparently he did not find any similar flagellates in 5 *M. lasiotis* or 5 *M. irus* [=*cynomolgus*]. He adds that his embadomonads were "successfully cultured [*sic*] on the R.E.S. medium", but gives no other details about their cultivation.

Hegner and Chu (1930) merely note that they found "small flagellates... that resembled *Embadomonas intestinalis*" in wild specimens of *M. philippinensis*; while Wenrich only records the finding of "*Retortamonas* (*Embadomonas*)" in a single individual of *M. rhesus* at Philadelphia. More recently Hegner (1934) has reported *Retortamonas* [=*Embadomonas*] from the faeces of captive chimpanzees in the United States.

No accurate study has yet been made—or, at least, published—of the embadomonads living in any species of monkey. The specific identity or diversity of them all is therefore still in doubt. In the course of my own work I have encountered *Embadomonas* in no primate except Man, and all my experimental macaques were naturally uninfected[2]. I have therefore been able to use them in attempts to transmit the flagellate of Man to monkeys experimentally.

(2) *Nomenclature*

I discussed the nomenclature of *Embadomonas intestinalis* briefly in 1921, and at that date it appeared to be satisfactorily settled[3]. Recently, however, new difficulties have arisen, so I must now refer to the subject once more.

Embadomonas intestinalis was discovered (in Man, in Egypt) by Wenyon and O'Connor (1917), who described it under the new generic name of *Waskia*. It soon became evident, however, that this was a synonym of *Embadomonas* Mackinnon, 1911 (*emend.* 1915); and to this genus the flagellates of Man were, accordingly, assigned during the next decade—from 1921 to 1932. Evidence was then brought forward by Wenrich (1932) to show that *Embadomonas* Mackinnon, 1915, is a synonym of *Retortamonas* Grassi, 1879: and in pursuance of this interpretation he made a drastic recension of the whole range of organisms

[1] Redescribed later: see Fonseca (1920).

[2] Evidence for the non-infection of the macaques employed in my experiments is given later.

[3] Cf. Dobell and O'Connor (1921), p. 79 *sq.* As full references to earlier papers were given in this work, I need not repeat them here.

previously known as *Retortamonas*, *Plagiomonas*, *Waskia*, and *Embadomonas*—most of which he referred to the first genus. Wenrich's judgement has already been accepted by several American authors.

Grassi's early genera of intestinal flagellates are notoriously difficult to interpret, and I have studied his papers with no better success than other protozoologists[1]. I can form an opinion of his genus *Retortamonas*[2] only from his own imperfect descriptions, as I have never seen any organisms like them. According to Grassi, *Retortamonas* (=*Plagiomonas*, the only species being *R. grillotalpae*) is a "very rare" intestinal flagellate living in larvae of the mole-cricket (*Gryllotalpa*) at Rovellasca (Italy). It has "the shape of a retort" (Grassi, 1882), and "2 anterior flagella, directed forwards, and 1 posterior (caudal)"—according to the redescription of *Plagiomonas* (Grassi, 1888). Although he was originally in doubt about the number of flagella, he ultimately stated categorically that *Retortamonas* was not—as Bütschli had suggested—a biflagellate like *Heteromita* or *Bodo*. In most other ways also Grassi's accounts and pictures of his *Retortamonas* differ materially from *Embadomonas*, as defined by Mackinnon and later workers. These genera, as defined by their authors, have, indeed, almost nothing in common.

It is thus possible to identify *Retortamonas* with *Embadomonas* only by rejecting almost the whole of Grassi's descriptions, on the grounds that he was entirely wrong: but the circumstance that Wenrich has found a typical *Embadomonas* in (? adult) mole-crickets in the United States is surely no proof of his errors. Consequently, though I have no personal knowledge of *Retortamonas* [=*Plagiomonas*] and am not prepared to define this genus in modern terms[3], I am satisfied that Wenrich's evidence is insufficient to warrant his conclusion; and I therefore consider that the "correct" name of the minute biflagellate organism living in the human intestine is not *Retortamonas* but *Embadomonas intestinalis*. Everybody will agree that it is most undesirable to change the name of any well-known "parasite" of Man without adequate reason: and it seems obvious, in the present case, that no evidence yet exists to make such a change imperative.

Faust and Wassell (1921) and Faust (1922) have described a second species of *Embadomonas* from Man—in China—under the name of *E. sinensis*: and the existence of this separate species has recently been upheld by Watt (1933). Moreover, Hogue (1933) has found what she believes to be a new variety (not named) of *E. intestinalis*—differing in several minor characters from the type—in a patient at Philadelphia. After reading all these various descriptions I am still unconvinced that more than one species of *Embadomonas* lives in the intestine of Man. I agree with Wenyon (1926) that *E. sinensis* is probably a

[1] I have studied them all with care: but I must note, as Bishop (1934) has already indicated, that Wenrich has seemingly overlooked Grassi's important final paper (1888) altogether.

[2] In 1882 Grassi changed the name to *Plagiomonas*—unjustifiably, according to the present Rules of Nomenclature, which did not then exist.

[3] I agree in the main, however, with the opinions expressed by Wenyon (1926) and Bishop (1932, 1934) regarding the interpretation of the genus.

synonym of *E. intestinalis*: and though variations within this species doubtless occur, I do not feel satisfied that the existence of any definitely recognizable and constant variety has yet been demonstrated.

I may add that, in my experience, "double individuals" often occur in faeces and in cultures—as with *Enteromonas*. These double forms do not divide immediately, but remain double—with 2 resting nuclei, 2 sets of flagella, and 2 mouths—for long periods. If I understand him correctly, Fonseca (1920) regards such organisms as the normal forms of "*Waskia*", which he apparently regards as distinct from *Embadomonas*. But his words are ambiguous, and in any case it is certain that the "monozoic" and "diplozoic" individuals of *Embadomonas* both belong to the same genus—whatever it be called.

(3) *Cultivation*

Embadomonas intestinalis (from Man) was first cultivated by Hogue (1921), who called the organism *Waskia*. The same worker has since published (Hogue, 1933) further details, though she now refers the flagellate to the genus *Retortamonas*.

Since 1921 many other workers have succeeded in cultivating *E. intestinalis* in a variety of media. It is readily cultivable by many methods, and has proved to be the easiest of all the intestinal protozoa of Man to isolate and maintain *in vitro*. In all media of the solid-liquid type invented by Boeck it grows abundantly—with suitable bacteria—and forms cysts copiously. I have successfully used all the media described in an earlier paper[1], but prefer that designated "HSre"—a slope of inspissated whole horse-serum, covered by egg-albumin diluted with Ringer's fluid.

Most of my own observations have been made on a strain (*G.*) which I obtained by accident. As I have used it for many experiments I add the following details:

Strain G.—In January 1932 I examined, by request of his medical adviser, the stools of a native of Britain who was suspected of infection with *E. histolytica*. The patient had travelled extensively, in the tropics and elsewhere, and had suffered from dysentery in the past; but at the date of my examination he was suffering from no intestinal disorder, and I found no pathogenic protozoa in his faeces. One of my cultures made on 26. i. 32, however, produced an abundant growth of *Embadomonas*—mixed with amoebae and *Blastocystis*. By the usual methods I succeeded in isolating the flagellates in "pure" culture—free from all other protozoa and *Blastocystis*—and I have maintained them *in vitro* as Strain *G.* (or its derivatives) ever since.

This strain grew luxuriantly in all the media which I generally use for intestinal protozoa. The main line (*G.*) I studied in some detail, but I abandoned it finally on 13. iv. 34 after continuous cultivation for over 2 years. It was then

[1] Dobell and Laidlaw (1926*a*), p. 294. Solid starch is an unnecessary ingredient, however as *Embadomonas* cannot ingest it.

growing excellently in "HSre" medium at 37° C.—in which transplantation was necessary only once a week—and could have been continued *ad libitum*.

While studying this strain I "discovered" (for I found afterwards that it had already been noted by several previous workers) that *E. intestinalis* is cultivable at ordinary room-temperatures. It not merely survives but grows, divides, and encysts freely at 15° C. and all temperatures between this and 38° C. At the lower temperatures it grows more slowly, but otherwise its development appears to be identical. The only difference I have noted is that much larger flagellates often occur in cultures kept at room-temperatures (*ca.* 15°–20° C.) than in those incubated at 37° C., though the cysts are always of the same size. Even at 10° C. the flagellates can live for long periods, but they make no growth. Cultures cannot be propagated, therefore, at this or any lower temperature.

Strain *G.*, or its derivatives, grows so well at the ordinary temperatures of my laboratory that I have latterly maintained it solely in this manner. I keep the cultures, in "HSre" medium, on my bench—regardless of daily fluctuations in temperature (which has ranged at times from below 10° C. to over 25° C.)—and make subcultures only once a month[1]. But the cultures often live for 2 months, or even longer (as compared with 1 week—up to 2—at 37° C.). I have not yet determined the maximum time for which they can live, but I have found living flagellates still present—and discoverable microscopically as well as culturally—for all periods up to 9 weeks (64 days).

Although Strain *G.* encysted freely and regularly in my cultures, I never succeeded in hatching the cysts *in vitro*. On incubation, in all media tried, they invariably degenerated and died. Many times, it is true, I have thought that I had obtained cultures from cysts: but I have always convinced myself afterwards that such cultures were really derived from flagellates accidentally accompanying them. Whenever I have inoculated living cysts alone—certainly freed, by chemical reagents or other means, from all possible contamination with active forms—I have never once obtained any growth of flagellates in culture-tubes containing any medium incubated at any temperature. I still do not know, therefore, how to hatch the cysts of *E. intestinalis*—save by feeding them to a suitable host—and I can find no indication in the publications of others that they have succeeded in any similar attempts.

In order to show the ease with which *E. intestinalis* can survive and be grown at temperatures far below that of the human body, I may add the following anecdotes—already partly recorded elsewhere[2]. On 24. v. 32 I sent 2 living samples of Strain *G.*, sealed in small glass tubes, to my friend Dr Toynbee Wight in California. I sent them by ordinary letter-mail, and he received them a fortnight later. From this material he obtained excellent cultures; and

[1] I do this not because it is necessary but because I have found a month an absolutely safe interval and one easy to remember—a not unimportant consideration when one has to maintain numerous strains of various protozoa single-handed for long periods.

[2] See Bishop (1934), and the annual *Report of the Medical Research Council* for 1932–3.

on 11. vii. 32 he sent back—by post again, without any special precautions—similar specimens to me in London. I received them 11 days later (on 22. vii. 32), and recovered the flagellates in subcultures without difficulty. I have kept this substrain of *E. intestinalis* (designated *GW.*) ever since; and it is now, at the time of writing (May 1935), at its 90th generation and still growing excellently after crossing the Atlantic Ocean twice. Since 1932, moreover, it has successfully made 2 further passages. In September 1933 I sent it to Miss M. J. Hogue at Philadelphia, and she received it safely and was able to continue it. As she lost the strain later, however, I afterwards mailed her another specimen in September 1934, at her request; and this she also received safely and has since been able to propagate successfully—as she kindly informs me.

Substrain *GW.* (derived from *G.*) has been described in detail by Miss A. Bishop (1934), to whom I entrusted it for special study. As her observations on its morphology, division, and encystation have already been published, I need add no further particulars here. I shall now only record a couple of experiments which I have been able to make with it.

(4) *Attempts to transmit* Embadomonas *from Man to Macaques*

I have been able to make only 2 attempts to infect monkeys with *Embadomonas* from Man. They were made with Strain *GW.* of the flagellates—just described[1]—and my tame macaques Rosa and Susanna, who have been used for many other experiments partly recorded elsewhere[2]. The data of present importance are as follows:

(i) **Susanna**, *M. sinicus* ♀. I fed this monkey on all the cysts in a rich culture of Strain *GW.* (52nd serial generation) on 13. ii. 33. The culture, in "HSre" medium, was inoculated on 8. ii. 33, incubated at 37° C. for 4 days, then kept at room-temperature for a day, during which time the abundant cysts were washed and concentrated by sedimentation in sterile half-strength Ringer's fluid. They were administered in a little pasteurized milk—to make the dose palatable—and all readily swallowed.

Before this experiment was made, Susanna had been under my continuous observation for over 8½ years. During this period she had never been in contact with any other animal infected with *Embadomonas*; and I had examined her stools from 24. vii. 24 to 12. ii. 33 altogether 446 times microscopically and on 355 occasions also culturally. Many of these examinations were very thorough, but *Embadomonas* was never discovered. I am confident, therefore, that this macaque was uninfected at the date of the experiment.

After the feeding, I examined Susanna's faeces daily—both microscopically and culturally—for the next 32 days (14. ii. 33–17. iii. 33): and since then at irregular intervals till the time of writing (May 1935). In all, I have made 68 examinations since 13. ii. 33—all completely negative for *Embadomonas*.

[1] *Vide supra.*

[2] See the earlier instalments of this series, especially Dobell with Bishop (1929) and Dobell (1931, 1933, 1934).

As this macaque is infected with *Enteromonas*, which almost always develops abundantly in primary cultures made from her faeces, I took special precautions to overcome this possible source of error: for it would be extremely easy to overlook a few embadomonads in a rich culture of enteromonads—even by the most careful microscopical examination. Accordingly, I kept most of the cultures negative for *Embadomonas*, but usually containing abundant *Enteromonas*, at room-temperature for several days in order to kill all the latter: and I then inoculated subcultures from the cooled tubes, and incubated and examined them at appropriate times. Neither of these flagellates ever developed in such attempted subcultures, though *Embadomonas* certainly would have done so had it been present.

I am therefore confident that Susanna was not infected—even temporarily[1]—with *Embadomonas* as a result of my experiment. She was negative for over 8½ years previously, and has remained equally negative despite intensive study for over 2 years since.

(ii) **Rosa**, *M. rhesus* ♀. I made a similar experiment with this monkey on 8. iv. 33, when I fed her on washed culture-cysts of *Embadomonas* (Strain *GW.*, 65th serial generation, in "HSre" medium) administered in the same way. The culture had been previously incubated for 4 days at 37° C., but the cysts had then been kept at room-temperature for the next 3 days.

Rosa had been under continuous observation before this experiment for over 6 years; and during this period her faeces had been examined 456 times microscopically and 443 times culturally[2]—always with completely negative results so far as *Embadomonas* is concerned. She was undoubtedly, therefore, a "clean" animal at the time. Precautions similar to those noted in the case of Susanna were, of course, taken throughout.

After the experimental feeding I examined Rosa's faeces daily—both microscopically and culturally—for 3 weeks (8. iv. 33 to 29. iv. 33), and afterwards intermittently on various dates. To the time of writing (May 1935) I have made 69 examinations in all, and they have all been completely negative.

It seems certain, therefore, that this monkey—like Susanna—has acquired no infection from ingesting the cysts of *Embadomonas intestinalis*. The data appear to me conclusive. They show that neither Susanna (*M. sinicus*) nor Rosa (*M. rhesus*) is readily infectible, by "pure" cultures administered by the natural route, with at least one strain of the flagellates of Man.

It should be added that neither of these monkeys suffered the least harm from my experiments, and that both had been previously treated several times with emetine[3]. But it is highly improbable that this could have influenced the

[1] The importance of careful control by cultural methods, in such experiments, has already been shown in the case of *Trichomonas*: cf. Dobell (1934). I was therefore fully alive to all the possibilities of transient infection.

[2] As noted elsewhere (Dobell, 1934), about 100 of the earlier examinations were made by Miss A. Bishop. I alone am responsible for the rest.

[3] Cf. Dobell (1931).

present experiments in any way, for Susanna's last treatment ended on 10. x. 29, and Rosa's on 11. x. 29—nearly 3½ years before the attempts were made.

(5) *Discussion*

I believe that the intestinal flagellates of Man hitherto described under the names of *Embadomonas*, *Waskia*, or *Retortamonas*, are probably all of one species: and it seems likely that the Macaques naturally harbour similar organisms. But as no accurate account of the embadomonads discovered in divers monkeys by various workers has yet been published, it is not possible—at present—to say with certainty that any *Embadomonas* of any macaque is, or is not, identical with *E. intestinalis* of Man.

I hoped to be able to shed some light on this matter by infecting my own macaques experimentally with flagellates of human provenance; but unfortunately I have hitherto been able to make only 2 such experiments, and both yielded negative results. I have proved—conclusively, I think—that a strain of *E. intestinalis* (from Man) was not transmissible to 1 individual of *M. rhesus* and 1 *M. sinicus* by feeding them on cysts from "pure" cultures: but such findings are obviously inadequate for any generalization.

Negative experiments such as I have made do not prove that *E. intestinalis* is not specifically identical with the *Embadomonas* which—according to the reports of others—occurs naturally in macaques[1]. They do show, however, that it is still unjustifiable to conclude that all simian flagellates resembling *E. intestinalis* are necessarily of this species. In the absence of other evidence it seems best to preserve an open mind on all such problems, and it is obviously rash—though now fashionable—to identify all the species of intestinal protozoa living in Man with superficially similar forms found in various other Primates.

The remarkable fact that *E. intestinalis* can be cultivated at ordinary room-temperatures at once suggests the possibility that it may be identical with similar species inhabiting cold-blooded animals. Clearly this can be determined by experiment alone; but the only experiments of any importance hitherto reported are those of Bishop (1934), who unsuccessfully attempted to infect tadpoles of the toad (*Bufo vulgaris*) by feeding them on cysts of *E. intestinalis* (my strain *GW.*). These cysts, as she remarks, "are strikingly similar in structure" to those of *E. dobelli*, which lives in English frogs and toads (Bishop, 1931); but her negative results indicate that this species is probably distinct from *E. intestinalis*.

At present, therefore, it is reasonable to conclude that *E. intestinalis*—despite its resemblance to other species—is a flagellate proper to Man, though *probably* capable of inhabiting some monkeys and *possibly* able to live even in cold-blooded vertebrates. Much further work is needed to delimit the species *E. intestinalis* definitively, to determine its exact distribution in nature, and to establish the various other "species" of *Embadomonas* which now figure in the

[1] Cf. my own results with the trichomonads of men and macaques (Dobell, 1934).

literature of protozoology. In the absence of all essential data, it is obviously best, for the present, to adopt a Pyrrhonian attitude and "suspend one's judgement" on all such questions.

Those writers who now confidently pronounce all flagellates in men and monkeys generally to be identical may prove to be right in the long run, but without more evidence such identifications are still mere predictions resting upon ignorance; and it should not be forgotten that in the laws of Nature—as in those of Man—"*non est interpretatio, sed divinatio, quae recedit a litera*"[1].

Summary and Conclusions

(A) *Enteromonas hominis* Fonseca, 1915 (*emend.* Dobell, 1921) is the correct designation of the flagellate first adequately described as *Tricercomonas intestinalis* by Wenyon and O'Connor in 1917.

Organisms morphologically indistinguishable, at all stages in the life-cycle, inhabit various macaques (*M. rhesus, M. sinicus, M. nemestrinus*). I have grown the simian species in "pure" cultures, and have obtained all stages *in vitro* and *in vivo*: and I have also obtained evidence—not "experimental", but from observed spontaneous spread of infection—that the species in *M. rhesus* and *M. sinicus*, at least, are identical.

An attempt to infect a man experimentally, by oral administration of cysts from a pure culture of the simian species (from *M. sinicus*), was unsuccessful.

(B) *Embadomonas intestinalis* [=*Retortamonas* Wenrich *nec* Grassi] is probably the correct designation of the flagellate described by Wenyon and O'Connor (1917) as *Waskia*: and it is the only intestinal protozoon of Man which can be readily cultivated at all temperatures from 15° to 38° C. This species closely resembles several others described from other vertebrates—both warm-blooded and cold-blooded—but the identity of them all is still questionable.

A species similar to *E. intestinalis* has been observed in macaques by several workers, and has been assumed to be identical on insufficient evidence. Two attempts which I have made to infect macaques (*M. rhesus* and *M. sinicus*) experimentally, by feeding on cysts in pure cultures of *E. intestinalis* from Man, were completely negative.

(C) Consequently, while it seems probable on general grounds that both the *Enteromonas* and the *Embadomonas* of men and macaques are specifically identical, it is still premature—with only negative evidence for their transmissibility from host to host—to assert this conclusion as a fact.

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A PARASITIC COPEPOD, *PUPULINA FLORES*, REDESCRIBED AFTER FORTY YEARS

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(With 13 Figures in the Text)

In 1892 P. J. van Beneden described and figured among some caligids from Africa and the Azores a new genus and species to which he gave the name *Pupulina flores*. He claimed to have found both sexes of the new parasite, but acknowledged that he had described as male and female of his species two copepods which had previously been designated by different names. They were found upon the same host, a devil-fish, and he believed that they were of the same species. In this, however, he was mistaken, for the specimen he described as the male was really a young female of an entirely different species. Owing to this mistake, together with the very imperfect description of both sexes, Bassett-Smith in 1899 denied the validity of the new genus and transferred Beneden's species to the well-known genus *Lepeophtheirus*. Following this lead the present author placed it in a key to the species of *Lepeophtheirus* published in 1905, and it has remained there up to the present time.

The identity of Beneden's "male" has always been doubtful because of the incomplete knowledge of its development. Recently, however, a collection of parasites from fish in the region of the Dry Tortugas was found to contain many specimens of *Paralebion elongatus*. Among these were two young females which were found to correspond in every particular with Beneden's description and figures. From this it appears that his "male" was simply a young female of this species.

But the female *Pupulina* really represented a new genus and species as Beneden claimed, and its validity has been established by the discovery of new specimens. During the Gifford Pinchot Expedition to the Galapagos Islands in 1929 twelve specimens of a copepod parasite were taken from the outer surface of a captured devil-fish, *Manta birostris*, the same species of fish as that from which Beneden's specimens were collected. Both sexes were included in this collection, and upon examination the males proved to be the true males of the species and very different from the one described and figured by Beneden. Accordingly both sexes of the new genus can be recognised and clearly distinguished from other caligids.

Genus **Pupulina** P. J. van Beneden

Female. General form like that of *Lepeophtheirus*; first three thoracic segments fused with the head; frontal plates well defined and without lunules. Fourth segment free, without dorsal plates; genital segment moderately enlarged

and its posterior corners produced; abdomen narrow and elongate, more or less distinctly two-segmented; caudal rami linear and as long as the entire abdomen. First antennae two-segmented; second pair stout, uncinate; prehensile hooks present behind the antennae; first maxillae straight, spines undivided at the tip; maxillipeds uncinate; no furca. Three pairs of biramose legs, rami of first pair two-segmented, of second and third pairs three-segmented. Fourth legs uniramose and four-segmented; no leg rudiments on the genital segment.

Male. Cephalothorax similar to that of the female and longer than the rest of the body; fourth segment free and relatively longer than in the female. Genital segment no wider than the fourth segment, narrowed posteriorly, with conspicuous spermatophore receptacles and minute processes at the posterior corners. Abdomen narrow, elongate, three-segmented, caudal rami linear and as long as the entire urosome, their inner margins fringed with setae. First antennae like those of the female, second pair with accessory spines; prehensile hooks greatly enlarged; first maxillae bipartite at their tips; the other mouthparts and the swimming legs like those of the female.

Pupulina Flores P. J. van Beneden (1892)

Occurrence. Twelve specimens, including both sexes, were taken from the outer surface of a "sea devil", *Manta birostris*, near the Galapagos Islands during the Gifford Pinchot Expedition in 1929. The best specimens of male and female were selected and given Cat. No. 60438 U.S.N.M. The male will serve as the original type of that sex, and since Beneden's specimens have been lost the female may be considered as a substitute for his type female.

Female. Carapace orbicular, as wide as long, the anterior margin convex, with a slit-like median sinus, the posterior margin nearly straight. Posterior sinuses slightly inclined, median lobe less than half the entire width, lateral lobes nearly straight. The pattern of the grooving on the dorsal surface of the carapace is shown in Fig. 1, its most noticeable feature being the two transverse grooves across each lateral area. Free segment two-sevenths of the width and only one-tenth of the length of the carapace, with no trace of dorsal plates. Genital segment half as long and wide as the carapace, with straight sides and sharp conical lobes at the posterior corners. Beneden represented the genital segment of the female as covered with spines, most thickly scattered along the lateral margins, but on the present specimens they are quite inconspicuous and visible only in profile.

The abdomen is conical, three times as long as its basal width, and more or less distinctly two-segmented, the basal segment more than twice the length of the terminal one. The caudal rami are flattened laterally and so closely juxtaposed as to be practically in contact along their inner surfaces. They are as long as the abdomen, their dorsal margins are fringed with short hairs, and each carries a tuft of small setae at its tip. The ovisacs are slender and about as long as the entire body; the spermatophores are lunate with a very short neck.

The first antennae are slender, the basal segment densely setose, the terminal segment rod-like with setae at the tip only. The second antennae are stout and their terminal claws are long and bent into a half circle. The basal segment carries a long and sharp spine on its ventral surface, pointed backward.

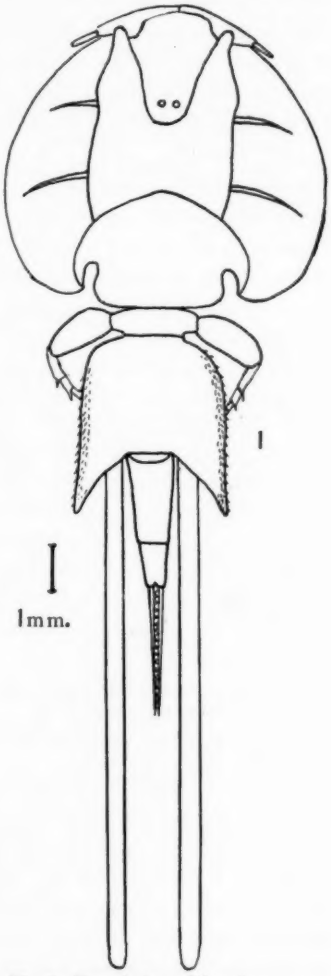

Fig. 1. Dorsal view of female.

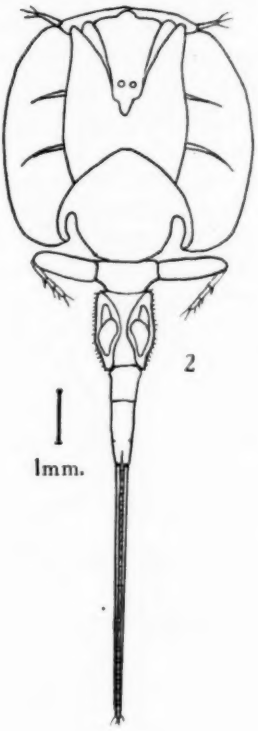

Fig. 2. Dorsal view of male.

The prehensile hooks are well developed and strongly curved; they are outside the second antennae and some distance behind the frontal margin. The first maxillae are simple spines, undivided at their tips, and the maxillary palps are also spines bearing a seta on their base. The second maxillae are rather slender, the terminal joint as long as the basal and tipped with two unequal

setae. The maxillipeds are small and weak with a short terminal claw, which is scarcely half the length of the basal segment. There is no furca but there are three pairs of spines on the ventral surface pointed backward, which might well serve the same purpose.

The rami of the first legs are two-segmented, the proximal segments unarmed, the distal exopod segment with four terminal claw-like spines and three large plumose setae on the inner margin. The distal endopod segment is curved inward and enlarged at its tip into a spherical knob, which is armed with three plumose setae curved inward and backward. The rami of the

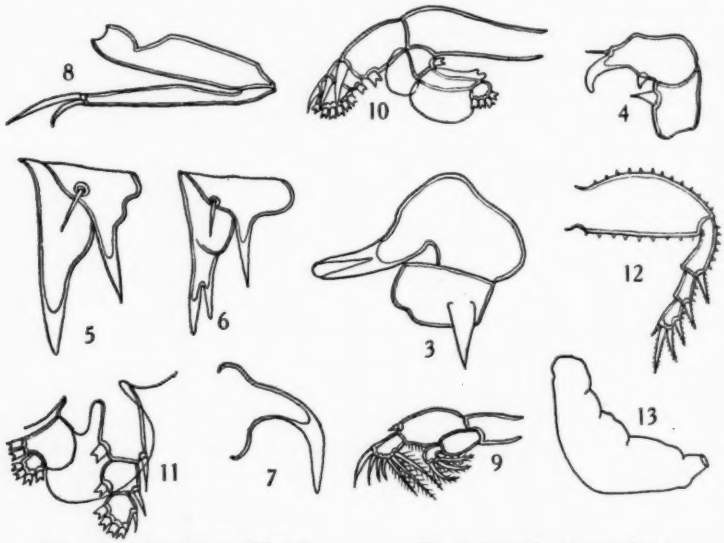

Fig. 3. Second antenna of female.
Fig. 4. Second antenna of male.
Fig. 5. First maxilla of female.
Fig. 6. First maxilla of male.
Fig. 7. Prehensile hook of male.
Fig. 8. Second maxilla of female.
Fig. 9. First leg of female.
Fig. 10. Second leg.
Fig. 11. Third leg. Fig. 12. Fourth leg. Fig. 13. Spermatophore.

second and third legs are three-segmented, each segment of the exopod with an outer spine, the two basal segments with an inner seta, the end segment with six setae in the second leg and four setae in the third leg. The basal segment of the second endopod has an inner seta and an outer lamina, the second segment has no seta but has both an outer and an inner lamina, the latter narrow and irregular at its distal end. The end segment is almost spherical and is armed with four setae, but has no lamina. The basal segment of the third endopod has no seta, but carries a large outer lamina which reaches distally to the tip of the ramus, and outwardly covers most of the two basal segments of the exopod. The second endopod segment has two inner setae and a small outer lamina, the third segment is a flattened sphere with four setae.

The basal segment of the fourth leg is stout and sparsely fringed with minute spines, the second, third and fourth segments are slender with an outer fringe of similar minute spines. The second and third segments each carry a single large spine at the outer distal corner, the fourth segment has two equal spines on its outer margin and two very unequal ones at its tip. All these large spines are denticulate on both margins. There are no rudiments of either a fifth or sixth pair of legs.

Total length, without the caudal rami, 13 mm., with the rami 16 mm. Length of carapace 6·80 mm., width 7·20 mm. Length of ovisacs 11·50 mm.

Male. Considerably smaller than the female, carapace orbicular and with the same dorsal grooving. Free thoracic segment as wide as the genital segment, without dorsal plates, narrowed anteriorly and posteriorly and widened through the bases of the fourth legs. Genital segment barrel-shaped, widest across the anterior margin with somewhat prominent anterior corners, its lateral margins convex, its posterior corners produced into minute rounded knobs. The spines along the lateral margins are more conspicuous than in the female. The abdomen is conical, longer than the genital segment and tapered posteriorly; it is indistinctly three-segmented, the division between the last two segments showing only on the ventral surface. The caudal rami are as long as the entire body behind the carapace and very slender.

The second antennae are shorter and stouter than those of the female and the terminal claws are armed with accessory spines. The first maxilla is bipartite at its tip, the other mouthparts and the swimming legs are like those of the female.

Total length 10·20 mm. without the caudal rami, 15 mm. with them. Carapace 5·70 mm. long, 5·60 mm. wide.

Remarks. This species can be recognised at once by the extremely long and slender caudal rami in both sexes, and by the prominent posterior processes at the corners of the genital segment of the female. The structure of the first maxillae, the swimming legs and the caudal rami, and the absence of a furca and of leg rudiments on the genital segment are the distinctive characters. The dorsal pattern of the carapace is also quite different in type from that of other caligids. The huge ray (20 feet in diameter) which was the host for this parasite, is seldom captured by a collecting expedition, and this explains the long interval between Beneden's original discovery and this first recurrence of the species.

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INDEX OF AUTHORS.

PAGE

BISHOP, ANN. Observations upon a "*Trichomonas*" from Pond Water. (With Plate VII) 246

BISHOP, ANN. Observations upon *Chilomastix* from *Bufo vulgaris*, with notes on *Chilomastix aulastomi*. (With Plate XVII, containing Figs. 1–32) 507

BUXTON, P. A. Changes in the Composition of Adult *Culex pipiens* during Hibernation. (With 1 Figure in the Text) 263

BUXTON, P. A. The Effect of *Proteosoma* upon the Survival of *Culex* 547

CARR FRASER, W. A. The Comparison of the Efficiency of Anthelmintics . . . 465

COLE, H. A. On some Larval Trematode Parasites of the Mussel (*Mytilus edulis*) and the Cockle (*Cardium edule*). (With 5 Figures in the Text) 276

DOBELL, CLIFFORD. Researches on the Intestinal Protozoa of Monkeys and Man. VII. On the *Enteromonas* of Macaques and *Embadomonas intestinalis* . . 564

DUKE, H. LYNDHURST. On *Trypanosoma brucei*, *T. rhodesiense* and *T. gambiense* and their Ability to Infect Man 46

DUKE, H. LYNDHURST. Further Studies of the Behaviour of *T. rhodesiense*, recently Isolated from Man, in Antelope and other African Game Animals . . . 68

EVANS, A. C. Studies on the Influence of the Environment on the Sheep Blow-fly, *Lucilia sericata* Meig. II. The Influence of Humidity and Temperature on Prepupae and Pupae. (With 6 Figures in the Text) 291

EVANS, A. C. Studies on the Influence of the Environment on the Sheep Blow-fly, *Lucilia sericata* Meig. III. The Influence of Humidity and Temperature on the Adult. (With 4 Figures in the Text) 299

GWYNN, A. M. and HAMILTON, A. G. Occurrence of a Larval Cestode in the Red Locust *Nomadacris septemfasciata* Serv. (With 4 Figures in the Text) . . 551

HAPPOLD, FRANK CHARLES and STEPHENSON, DORA. The Toxicity of Bactericidal Substances for Flagellate Parasites, with Special Reference to their Application in the Isolation of *Leptomonas* Forms 383

HOLMES, FRANCIS O. Method of Chain Formation in *Herpetomonas bancrofti*. (With 9 Figures in the Text) 394

HOPKINS, G. H. E. Some Observations on the Bionomics of Fleas in East Africa . 480

IYENGAR, M. O. T. Two New Fungi of the Genus *Coelomomyces* Parasitic in Larvae of *Anopheles*. (With 5 Figures in the Text) 440

JOHRI, L. N. On Cestodes from Burma. (With 4 Figures in the Text) . . . 476

KEILIN, D., TATE, P. and VINCENT, M. The Perispiracular Glands of Mosquito Larvae. (With 2 Figures in the Text) 257

KIRTISINGHE, P. Parasitic Copepods of Fish from Ceylon. (With 46 Figures in the Text) 332

KRULL, WENDELL H. Studies on the Life History of *Panopistus pricei* Sinitsin, 1931 [Trematoda]. (With Plate I, containing Figs. 1–7) 93

PAGE

LAPAGE, GEOFFREY. The Second Ecdysis of Infective Nematode Larvae. (With 6 Figures in the Text) 186

LAWRENCE, R. F. The Prostigmatic Mites of South African Lizards. (With 22 Figures in the Text) 1

LEIGH-SHARPE, W. HAROLD. *Epibrachiella impudica* (Nordmann) (Copepoda). (With 8 Figures in the Text) 101

LEIGH-SHARPE, W. HAROLD. Some New and Rare Lernaeidae (Copepoda) from Plymouth. (With 2 Figures in the Text) 107

LEIGH-SHARPE, W. HAROLD. *Anchistrotos laqueus* n.sp. a Parasitic Copepod of *Serranus cabrilla*. (With 3 Figures in the Text) 266

LEIGH-SHARPE, W. HAROLD. Two Copepods (*Lernaeenicus*) Parasitic on *Clupea*. (With 8 Figures in the Text) 270

MCINTOSH, ALLEN. Description of a tick, *Ornithodoros coprophilus* n.sp., from Bat Guano. (With Plate XVIII and 2 Text-figures) 519

MACLEOD, JOHN. *Ixodes ricinus* in Relation to its Physical Environment. II. The Factors Governing Survival and Activity. (With 8 Figures in the Text) . . 123

MACLEOD, JOHN. *Ixodes ricinus* in Relation to its Physical Environment. III. Climate and Reproduction. (With 2 Figures in the Text) 489

MANTER, H. W. The Structure and Taxonomic Position of *Megasolena estrix* Linton 1910 (Trematoda) with Notes on Related Trematodes. (With 7 Figures in the Text) 431

MARSHALL, J. F. and STALEY, J. Some Adult and Larval Characteristics of a British "Autogenous" Strain of *Culex pipiens* L. 501

MELLANBY, KENNETH. A Comparison of the Physiology of the Two Species of Bed-bug which Attack Man. (With 3 Figures in the Text) 111

MELLANBY, KENNETH. The Structure and Function of the Spiracles of the Tick, *Ornithodoros moubata* Murray. (With 2 Figures in the Text) 288

PATTEN, RUTH. The Life History of *Merocystis kathae* in the Whelk, *Buccinum undatum*. (With Plates XI–XIV, containing Figs. 1–56, and Figs. I–X in the Text) 399

PENTELOW, F. T. K. Notes on the Distribution of the Larvae and Pupae of *Simulium* spp. in the River Tees and its Tributaries. (With 1 Figure in the Text) . . 543

RAY, HARENDRANATH. On a New Coccidian, *Eimeria laminata* n.sp., from the Intestine of an Indian Toad, *Bufo melanostictus* Schneid. (With Plate X, containing Figs. 1–11) 369

ROTHSCHILD, MIRIAM. The Trematode Parasites of *Turritella communis* Lmk. from Plymouth and Naples. (With 22 Figures in the Text) 152

ROTHSCHILD, MIRIAM. Note on the Excretory System of *Cercaria ephemera* Lebour, 1907 (*nec* Nitzsch). (With 7 Figures in the Text) 171

SEN, S. K. The Mechanism of Feeding in Ticks. (With 15 Figures in the Text) . 355

SHARIF, M. On the Presence of Wing Buds in the Pupa of Aphaniptera. (With 2 Figures in the Text) 461

SHUTE, P. G. The Value of the External Spine of the Harpago in Identifying Varieties of *Anopheles maculipennis*. (With Plate XIX) 561

SKINKER, MARY SCOTT. A Redescription of *Taenia tenuicollis* Rudolphi, 1819, and its Larva, *Cysticercus talpae* Rudolphi, 1819. (With 2 Figures in the Text) . . 175

PAGE

SMITH, KENNETH, M. Two Strains of Streak: A Virus Affecting the Tomato Plant. (With Plates XV and XVI) 450

SMITH, KENNETH M. and BALD, J. G. A Description of a Necrotic Virus Disease affecting Tobacco and Other Plants. (With Plates V and VI and 2 Text-figures) 231

SMITH, KENNETH M. and DONCASTER, J. P. The Preparation of Gradocol Membranes and their Application in the Study of Plant Viruses. (With 7 Figures in the Text) 523

SRIVASTAVA, HAR DAYAL. New Parasites of the Genus *Orientophorus* n.gen. (Family Fellodistomidae) from an Indian Fresh-Water Fish, *Clupea ilisha*. (With 4 Figures in the Text) 374

TATE, P. The Larva of *Phaonia mirabilis* Ringdahl, Predatory on Mosquito Larvae (Diptera, Anthomyidae). (With 8 Figures in the Text) 556

TAYLOR, E. L. Do Nematodes assist Bacterial Invasion of the Host by wounding the Wall of the Intestinal Tract? 145

THEILER, HANS, AUGUSTINE, DONALD L. and SPINK, WESLEY W. On the Persistence of Eosinophilia, and on Immune Reactions in Human Trichinosis, Several Years after Recovery 345

THOMPSON, GORDON B. New Genera of *Mallophaga*. I. (With 4 Figures in the Text) 281

WERTHEIM, P. A New Ciliate, *Entodinium bovis* n.sp., from the Stomach of *Bos taurus* L., with the Revision of *Entodinium exiguum*, *E. nanellum*, *E. simplex*, *E. dubardi dubardi* and *E. parvum*. (With 6 Figures in the Text) 226

WILSON, CHARLES BRANCH. A Parasitic Copepod, *Pupulina flores*, Redescribed after Forty Years. (With 13 Figures in the Text) 593

WOODLAND, W. N. F. Some more Remarkable Cestodes from Amazon Siluroid Fish. (With Plates II–IV, containing Figs. 1–34, and Fig. I in the Text) 207

WOOLCOCK, VIOLET. Digenetic Trematodes from some Australian Fishes. (With Plates VIII and IX and 6 Figures in the Text) 309

INDEX OF SUBJECTS

PAGE

African Lizards, mites parasitic on 1

Anthelmintics, comparison of the efficiency of 465

ARACHNIDA

. ACARINA

. Prostigmata

. *Ixodiderma* n.gen. 22

. *inverta* n.sp. 24

. *lacertae* n.sp. 29

. *pilosa* n.sp. 30

. *Pterygosoma* 33

. *bicolor* n.sp. 34

. *gerrhosauri* n.sp. 33

. *hystrix* n.sp. 37

. *Scaphothrix* n.gen. 20

. *convexa* n.sp. 21

. *Zonurobia* n.gen. 4

. *circularis* n.sp. 8

. *cordylensis* n.sp. 5

. *debilipes* n.sp. 13

. *montana* n.sp. 19

. *polyzonensis* n.sp. 7

. *sanguinea* n.sp. 16

. *semilunaris* n.sp. 11

. *subquadrata* n.sp. 18

. *transvaalensis* n.sp. 15

. Ixodoidea

. *Ixodes ricinus*, in relation to its physical environment 123, 489

. mechanism of feeding in Ticks 355

. *Ornithodoros coprophilus* n.sp., from bat guano 519

. *moubata*, structure and function of the spiracles of . . . 288

. *papillipes* Birula, sucking apparatus in 359

Australian fishes, some digenetic Trematodes from 309

Bacillus suipestifer, in conjunction with nematodes in rabbits 147

Bed-bugs, physiology of the two species which attack man 111

Bos taurus L., a new ciliate, *Entodinium bovis*, from the stomach of . . 226

Bufo vulgaris, observations upon *Chilomastix* from, with notes on *Chilomastix aulastomi* 507

Burma, cestodes from 476

Chain formation in *Herpetomonas bancrofti* 394

Clupea, two copepods (*Lernaeenicus*) parasitic on 270

Clupea ilisha, new Trematode parasites from 374

Cockle (*Cardium edule*), some larval Trematode parasites of 276

PAGE

CRUSTACEA

. COPEPODA

. *Anchistrotos laqueus* n.sp., description of 266
. *Brachiella thynni* Cuvier (1830) 342
. *Epibrachiella impudica* (Nordmann), on the gills of *Trigla cuculus* . . . 101
. *Haemobaphoides ambiguus* T. Scott 107
. *Lernaeenicus encrasicholi* (Turton 1807), parasitic on *Clupea* 273
. *spratta* (Sowerby), parasitic on *Clupea* 270
. *Lernaeocera mulli* n.sp. 109
. *Lernaeolophus sultanus* Nordmann (1864) 334
. *Pennella diodontis* Oken (1816) 332
. *Pseudocycnus appendiculatus* Heller (1865) 336
. *armatus* Bassett-Smith (1898) 339
. *Pupulina flores*, redescribed after forty years 593
. *Saucissona* n.gen. 107
. *lumpi* T. Scott 107
. *sauciatonis* n.sp. 107
. *Thysanote appendiculata* Steenstrup and Lütken (1861) 343
Eosinophilia, persistence of, and on immune reactions in human trichinosis . . 345
Excretory system of *Cercaria ephemera* Lebour, 1907 (*nec* Nitzsch) 171
Fish from Ceylon, parasitic Copepods of 332
Fleas, some observations on the bionomics of, in East Africa 480

FUNGI

. *Coelomomyces* Keilin
. *anophelesica* n.sp., description of 447
. *indiana* n.sp., description of 446
. parasitic in larvae of *Anopheles* 440
Gerrhosauridae, mites parasitic on 33

INSECTA

. APHANIPTERA
. presence of wing buds in the pupa of 461
. *Ceratophyllus gallinae*, wing buds in the pupa of 463
. *Nosopsyllus fasciatus* (Bosc), wing buds in the pupa of 462
. *Xenopsylla brasiliensis*, bionomics of, in East Africa 480
. *cheopis*, bionomics of, in East Africa 480
. DIPTERA
. *Anopheles* larvae, two new fungi parasitic in 440
. *claviger* (*bifurcatus*) Mg., perispiracular glands of larvae of . . 257
. *maculipennis*, value of external spine of the harpago in identifying varieties of 561
. *Culex*, effect of *Proteosoma* upon the survival of 547
. *Culex pipiens*, L. adult and larval characteristics of a British "autogenous" strain of 501
. changes in the composition of adult, during hibernation . . 263
. perispiracular glands of larvae of 257
. *Lucilia sericata* Meig., influence of humidity and temperature on prepupae and pupae of 291
. influence of humidity and temperature on the adult of . . 299

PAGE

INSECTA
. DIPTERA
. *Orthopodomyia pulchripalpis*, perispiracular glands of larvae of 257
. *Phaonia mirabilis* Ringdahl, larva of, predatory on mosquito larvae . . 556
. *Simulium* spp., notes on the distribution of larvae and pupae of, in the river Tees and its tributaries 543
. *Theobaldia annulata*, perispiracular glands of larvae of 257
. HEMIPTERA
. *Cimex lectularius* 111
. *rotundatus* 111
. MALLOPHAGA
. *Rhynonirmus* n.gen. 281
. *helvolus* (Nitzsch) 1838 285
. *infuscatus* (Osborn) 1896 282
. *scolopacis* (Denny) 284
. ORTHOPTERA
. *Nomadacris septemfasciata* Serv., occurrence of a larval Cestode in . . . 551
Lizards, African, mites parasitic on 1
Man, and monkeys, researches on the intestinal protozoa of 564
Membranes, gradocol, preparation of, and their application in the study of plant viruses 523
Mites, prostigmatic, parasitic on African Lizards 1
Monkeys and man, researches on the intestinal protozoa of 564
Mosquito larvae, perispiracular glands of 257
preyed upon by larva of *Phaonia mirabilis* Ringdahl 556
Mussel (*Mytilus edulis*), some larval Trematode parasites of 276
Perispiracular glands of mosquito larvae 257

PROTOZOA
. CILIATA
. *Entodinium bovis* n.sp., description of 226
. *dubardi dubardi*, revision of 226
. *exiguum*, revision of 226
. *nanellum*, revision of 226
. *parvum*, revision of 226
. *simplex*, revision of 226
. FLAGELLATA
. *Chilomastix aulastomi*, from *Bufo vulgaris* 509
. *Embadomonas intestinalis* 564
. *Enteromonas*, researches on 564
. *hominis*, researches on 566
. *Herpetomonas bancrofti*, method of chain formation in 394
. *Leishmania ceramodactyli*, isolation of 384
. *tropica*, isolation of 384
. *Leptomonas culicidarum* A, isolation of 384
. *culicidarum* C, isolation of 384
. *lygaeorum*, isolation of 384
. *muscarum*, isolation of 384
. *oncopelti*, isolation of 384
. *parva*, isolation of 384
. *spp.* isolation of 384

PAGE

PROTOZOA

Trichomonas keilini n.sp., description of 249
Trypanosoma brucei, ability to infect man 46
gambiense, ability to infect man 46
rhodesiense, ability to infect man 46
behaviour of, in antelope and other African game animals 68
SPOROZOA
Eimeria laminata n.sp., from intestine of Indian toad 369
Merocystis kathae, life history of, in whelk 399
Proteosoma praecox, effect of, upon the survival of *Culex* 547
Serranus cabrilla, parasitised by a copepod, *Anchistrotos laqueus* n.sp. . . . 266
Spiracles of the tick, *Ornithodoros moubata* 288
Toad, a new Coccidian from the intestine of 369
Tomato plant, two strains of streak virus affecting 451
Trichinosis, human, immune reactions in 345

VERMES

CESTODA
from Amazon Siluroid fish 207
from Burma 476
occurrence of a larval, in the red locust, *Nomadacris septemfasciata* Serv. . 551
Amphilaphorchidinae, note on the 222
Amphoteromorphus parkarmoo n.sp. 214
Anthobothrium mandubé n.sp. 208
Aploparaksis kamayuta n.sp., description of 476
Cysticercus talpae Rudolphi, 1819, description of 183
Endorchis mandubé n.sp. 207
Haemonchus contortus, use of drugs against, in sheep 471
Hymenolepis bilharzii (Krabbe 1869), description of 478
globirostris Baer 1925, description of 478
Manaosia braçodemoça gen. et sp.n. 213
Monticellia piracatinga n.sp. 210
rugosa n.sp. 219
Monticelliinae, the species of 220
Nomimoscolex kaparari n.sp. 217
lenha 215
piracatinga n.sp. 211
Rudolphiella lobosa, note on 222
Rudolphiellinae, note on the 222
Taenia tenuicollis Rudolphi, 1819, redescription of 175
its larva *Cysticercus talpae* Rudolphi, 1819 183
NEMATODA
Nematodes and bacterial invasion of the host 145
second ecdysis of infective larva 186
Graphidium strigosum 146
Haemonchus contortus, second ecdysis of larva of 186
Ostertagia circumcincta, second ecdysis of larva of 186
Trichostrongylus retortaeformis 148
Trichostrongylus spp., second ecdysis of larvae of 186

PAGE

VERMES

. TREMATODA

. parasites of *Turritella communis* Lmk. from Plymouth and Naples . . . 152
. *Bucephalus mytili* n.sp., from the mussel 279
. *Cercaria ampelis* n.sp. 167
. *doricha* n.sp. 165
. *ephemera* Lebour, excretory system of 171
. *herpsyllis* n.sp. 164
. *nicarete* n.sp. 166
. *pythionike* n.sp. 155
. *ranzii* n.sp. 167
. *tenuans* sp.inq., and orange sickness of mussels 276
. *Dactylostomum gracile* n.gen. et n.sp. 315
. Digenetic Trematodes from some Australian fishes 309
. *Erilepturus tiegsi* n.gen. et n.sp. 320
. *Hapladena*, notes on 435
. *Helicometra bassensis* n.sp. 314
. *tenuifolia* n.sp. 310
. *Megasolena estrix* Linton 1910, structure and taxonomic position of . . 431
. *Orientophorus brevichrus* n.gen. et n.sp. from an Indian fresh-water fish . . 374
. *clupii* n.sp., from an Indian fresh-water fish 379
. *gangeticus* n.sp., from an Indian fresh-water fish . . . 377
. *ilishii* n.sp., from an Indian fresh-water fish 378
. *Otodistomum pristiophori* (*Distomum pristiophori* Johnston) 330
. *Panopistus pricei*, Sinitsin, life history of 93
. *Parahemiurus australis* n.sp. 317
. *Probolitrema antarcticus* n.sp. 325
. *philippi* n.sp. 323

VIRUSES

. disease, necrotic, affecting tobacco and other plants 231
. in plant, preparation of gradocol membranes and their application in the study of 523
. Tomato Streak, two strains of 450
Whelk, life history of *Merocystis kathae* in 399
Zonuridae, mites parasitic on 4

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TICKS. A MONOGRAPH OF THE IXODOIDEA
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CONTENTS

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PAGE

CARR FRASER, W. A. The Comparison of the Efficiency of Anthelmintics 465

JOHRI, L. N. On Cestodes from Burma. (With 4 Figures in the Text) . 476

HOPKINS, G. H. E. Some Observations on the Bionomics of Fleas in East Africa 480

MACLEOD, JOHN. *Ixodes ricinus* in Relation to its Physical Environment. III. Climate and Reproduction. (With 2 Figures in the Text) . . 489

MARSHALL, J. F. and STALEY, J. Some Adult and Larval Characteristics of a British "Autogenous" Strain of *Culex pipiens* L. 501

BISHOP, ANN. Observations upon *Chilomastix* from *Bufo vulgaris*, with notes on *Chilomastix aulastomi*. (With Plate XVII, containing Figs. 1–32) . 507

MCINTOSH, ALLEN. Description of a tick, *Ornithodoros coprophilus* n.sp., from Bat Guano. (With Plate XVIII and 2 Text-figures) . . . 519

SMITH, KENNETH M. and DONCASTER J. P. The Preparation of Gradocol Membranes and their Application in the Study of Plant Viruses. (With 7 Figures in the Text) 523

PENTELOW, F. T. K. Notes on the Distribution of the Larvae and Pupae of *Simulium* spp. in the River Tees and its Tributaries (With 1 Figure in the Text) 543

BUXTON, P. A. The Effect of *Proteosoma* upon the Survival of *Culex* . . 547

GWYNN, A. M. and HAMILTON, A. G. Occurrence of a Larval Cestode in the Red Locust *Nomadacris septemfasciata* Serv. (With 4 Figures in the Text) 551

TATE, P. The Larva of *Phaonia mirabilis* Ringdahl, Predatory on Mosquito Larvae (Diptera, Anthomyidae). (With 8 Figures in the Text) . . 556

SHUTE, P. G. The Value of the External Spine of the Harpago in Identifying Varieties of *Anopheles maculipennis*. (With Plate XIX) . . . 561

DOBELL, CLIFFORD. Researches on the Intestinal Protozoa of Monkeys and Man. VII. On the *Enteromonas* of Macaques and *Embadomonas intestinalis* 564

WILSON, CHARLES BRANCH. A Parasitic Copepod, *Pupulina flores*, Redescribed after Forty Years. (With 13 Figures in the Text) . . . 593

INDEX OF AUTHORS 598

INDEX OF SUBJECTS 601

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